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(57) Abstract

A novel expression system is provided comprising a DNA sequence containing transcriptional and translational signals that promote the over production of recombinant proteins both in bacterial hosts (e.g., *Escherichia coli*) and yeasts (e.g., *Saccharomyces cerevisiae*). The design of the expression system lends itself to a unique strategy which allows heterologous genes to be directly cloned at a position relative to the transcription/translation signals which is optimal for expression. Particularly provided are expression cassettes comprising a sequence of the invention combined with a purpose built series of plasmids wherein the utility and efficiency of the resultant expression vectors can be demonstrated to over produce protein, particularly phenylalanine ammonia lyase (herein abbreviated to PAL), in *E. coli* and *S. cerevisiae* to levels hitherto unattainable.

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BI-FUNCTIONAL EXPRESSION SYSTEM.

The present invention relates to novel promoter DNA, particularly a novel expression system comprising DNA having a sequence containing transcriptional and translational signals that promote the over production of recombinant proteins both in bacterial hosts (eg., Escherichia coli) and yeasts (eg., Saccharomyces cerevisiae); and to a novel cloning method that allows the insertion of a heterologous gene into a vector or expression cassette directly at the authentic translational start point of a promoter, with no deleterious changes being made to either the native 5'-UTR of a vector promoter or to the codons of the inserted gene; allowing production of that promoter DNA. The design of the expression system lends itself to this unique strategy which allows heterologous genes to be directly cloned at a optimal position relative to the transcription /translation signals.

Particularly provided are expression cassettes comprising a sequence of the invention combined with a purpose built series of plasmids wherein the utility and efficiency of the resultant expression vectors can be demonstrated to over produce protein, particularly that of phenylalanine ammonia lyase (herein abbreviated to PAL), in E. coli and S. cerevisiae to levels hitherto unattainable.

Although considerable progress has been made towards the development of expression systems for yeast (reviewed in Rose and Broach, 1990), the vectors lack the sophistication and versatility of their bacterial counterparts. Current vectors often contain many superfluous DNA sequences, which make them cumbersome and difficult to amplify and isolate in large quantities. The wealth of DNA present means that unique restriction sites are limited in number.

Yeast expression vectors are usually of the "sandwich" variety, whereby cloning sites are "sandwiched" between a homologous yeast promoter and transcriptional termination signals. The precise positioning of the cloning sites with respect to the authentic initiating codon (AUG) of the homologous yeast promoter represents something of a dilemma. If one chooses to place the cloning sites upstream to the AUG, then one inevitably disrupts the native 5'-untranslated region (5'-UTR) of the yeast promoter. Unavoidable

insertion of heterologous untranslated sequence elements containing a high proportion of G residues, or elements creating secondary structures or containing the inserted AUG in a sub-optimal nucleotide context, can have catastrophic effects on expression levels, regardless of the strength of transcriptional activation signals (Donahue and Cigan, 1988; Baim and Sherman, 1988). For example, Bitter and Egan (1984) reported 10 - 15 fold lower expression levels of a Hepatitis B surface antigen (HBsAg) gene, fused to a yeast glyceraldehyde-3-phosphate (GPD) gene promoter, but utilising the native HBsAg 5' flanking region, compared to HBsAg fused to a GPD promoter and utilising the GPD 5' flanking region.

The alternative is to position the cloning sites immediately 3' to the authentic AUG of the yeast promoter. However, this has its own concomitant problems. Care must be taken that the fusion is "in frame", while the non-authentic amino terminus of the expressed protein may have unpredictable effects on its biological activity and antigenicity. These last two points render such fusion proteins unsuitable for use as a pharmaceutical without modification.

Preferably cloning is directly from the authentic AUG initiation codon. However, there has been no reported instance of a native yeast promoter with a usable restriction site encompassing its translational start point and the artificial creation of one would inevitably disrupt the start codon or its nucleotide context. The alternative is the lengthy and expensive procedure of chemically synthesizing an oligonucleotide "bridge" fragment that reaches from a convenient restriction site in the promoter 5' to the translational start to a site 3' to the ATG in the coding region to be expressed. Such a procedure is not applicable to a routine, versatile cloning strategy.

A further disadvantage with currently available yeast expression vectors is that as they employ homologous yeast promoters containing powerful transcriptional activating sequences and they do not direct the efficient transcription or translation in bacterial hosts, such as E. coli (Ratzkin and Carbon, 1977; Struhl, 1986). Similarly, bacterial derived transcriptional/translational signals are inefficiently utilised in S. cerevisiae, if at all. Comparative expression studies of heterologous genes in E.coli and S.cerevisiae

therefore require the use of two separate vector systems.

A preferred aspect of the present invention describes how both the specificity and efficiency of a yeast promoter element, particularly that of S. cerevisiae, particularly that of PGK, can be changed to direct the high expression of heterologous genes in bacteria and yeasts.

A first aspect of the invention provides promoter DNA incorporating a structural gene starting position characterised in that the DNA has a unique SspI restriction site at the structural gene start position. A second aspect of the invention provides a novel cloning strategy ie. method, that allows the insertion of a heterologous gene into the expression cassette directly at the authentic translational start point of the promoter, with no deleterious changes being made to either the native 5'-UTR of the vector promoter or to the codons of the inserted gene; thus providing the promoter DNA of the first aspect.

A third aspect of the invention provides recombinant DNA comprising a yeast promoter sequence, particularly of S. cerevisiae, characterized in that the leader region of the promoter sequence is replaced with that of the replication protein 2 (REP2) gene (ORF C) of the yeast 2 μm plasmid (Hartley and Donelson, 1980). A preferred yeast promoter derived portion is that of the phosphoglycerate kinase (PGK) promoter and encompasses powerful upstream activating sequences (UAS) (Ogden et al., 1986), responsible for efficient transcription in S.cerevisiae. The sequences necessary for efficient transcription in E.coli reside in the REP2 derived portion of the hybrid promoter. Sequences necessary for efficient translation, both in S.cerevisiae and E.coli, also reside in the REP2-derived portion of the promoter.

A fourth aspect of the invention provides the promoter hybrid of the invention incorporated into an expression "cassette", in which a copy of the lacZ' gene, containing the multiple cloning sites of pMTL23, is preceded by the promoter, and followed by tandemly arranged, yeast gene-derived, transcriptional terminators.

In the cloning method of the second aspect (illustrated in Figure 1) promoter DNA incorporating a structural gene starting position eg. within an expression cassette, is modified using SDM by creating a

unique SspI restriction site at a structural gene start position. The position of this created site is such that the triplet sequence, ATG, corresponding to the translational start codon of the structural gene becomes ATA within the SspI recognition site AATATT. The heterologous gene to be inserted is similarly modified. In this case the nucleotide triplet corresponding to the translational start codon (eg., AUG, GUG, or UUG) is changed to CAG, while the triplet immediately 5' is changed to CTG. These changes correspond to the creation of a PstI restriction site, CTGCAG. The creation of the PstI, or equivalent site, can be conveniently performed simultaneously to isolation of the gene by utilising a mutagenic primer in a polymerase chain reaction (PCR) catalysed gene amplification procedure (Higuchi et al., 1988). The modified heterologous gene can be then digested with PstI restriction endonuclease and the 3' overhanging ends removed eg. by the 3' to 5' exo-nucleolytic activity of T4 DNA polymerase. The heterologous gene can then be excised using any of the restriction enzymes whose sites are present within the polylinker of the vector.

The net result of the actions of these DNA modifying enzymes is that the first base of the blunt-ended DNA fragment is the third nucleotide, "G", of its first codon. It is then ligated into the vector which has been digested previously with SspI and a restriction enzyme compatible with that used to excise the heterologous gene. Fusion of the vector promoter region (which ends in "AT") and heterologous gene (which begins in a "G") results in the recreation of the translational start, ATG.

The fourth aspect of the invention provides an expression system obtainable using the method of the invention such that overexpression of proteins is possible. A particular example of this is provided in the over expression of phenylalanine ammonia-lyase (PAL) gene from Rhodosporidium toruloides in both E. coli and S. cerevisiae. This is made possible by incorporating an expression cassette provided by the method into a purpose built, unique series of S.cerevisiae/ E. coli shuttle plasmids. Preferably every component of these shuttle plasmids is extensively modified to reduce the presence of superfluous DNA in the final vectors and to eliminate nucleotide sequence motifs corresponding to the restriction enzyme recognition sites of use in

the operation of the expression cassette. The levels of recombinant PAL attained in S. cerevisiae are significantly higher than that obtained using the PGK promoter alone. Whereas the PGK promoter alone fails to elicit the expression of PAL in E.coli, the levels of recombinant PAL obtained using the hybrid promoters are far in excess of those previously obtained using expression vectors designed for high expression in E. coli.

The DNA, cassettes, and organisms of the present invention will now be illustrated by reference to the following non-limiting Figures and Examples. Other variations falling within the scope of the invention will be apparent to those skilled in the art in the light of these.

FIGURES and SEQUENCE LISTING SEQUENCES:

Figure 1: shows the design of the novel cloning method which allows cloning to take place directly at the authentic translational start point of a promoter.

Figure 2: shows a comparison of sequences found 5' to the translational start codon in REP2 and PGK, compared to a consensus yeast sequence, the sequence found 5' to the neo gene and a consensus procaryotic promoter sequence.

Figure 3: shows how genes are inserted into the expression cassette using the SspI site.

Figure 4: shows an overview of the pMTL 8XXX vectors of the invention.

Figure 5: shows an SDS-PAGE electrophoretogram of lysates derived from microbial cells producing recombinant PAL.

Figure 6: shows the construction of pMTL 8000 and pMTL 8100 by inserting a 1.4 kb RsaI fragment from pVT100-U, containing the origin of replication and the STB locus from the 2 μm circle plasmid, into the EcoRV site of pMTLJ and pMTL CJ.

Figure 7: shows the construction of pMTLCJ by replacing the bla gene of pMTL4 with the cat gene of pCM4, modified by SDM-mediated removal of EcoRI, NcoI and SspI restriction sites.

SEQ ID No 1: shows the complete nucleotide sequence of a novel expression cassette (SEQ ID No 1) of the invention including a sequence comprising the PGK::REP2 promoter (bases 1-635; REP2 fragment consists of bases 547-635 of SEQ ID No 1).

SEQ ID No 2: is the complete nucleotide sequence of a comparative control cassette, containing the PGK promoter.

SEQ ID No 3: is the nucleotide sequence of plasmid pMTL8000.

SEQ ID No 4: is the nucleotide sequence of plasmid pMTL8100.

SEQ ID No 5: is the nucleotide sequence of the URA3-J and ura3-dJ (189 =G) alleles used in the vector construction.

SEQ ID No 6: is the nucleotide sequence of the leu2-dJ allele used in vector construction.

In SEQ ID No 1, the original nucleotide source DNA sequences have been changed by SDM at the following points:

Base 548-549	was TT	now AC	Creates ClaI::AccI
Base 557	was G	now T	
Base 580	was G	now T	
Base 636-638	was GTG	now ATT	
Base 1033-1035	was TAA	now GTT	Creates HpaI::BglII
Base 1149	was G	now C	Removes ClaI
Base 1223	was T	now A	Removes SspI
Base 1484	was G	now T	Removes AccI

Restriction endonuclease sites are provided in regions of DNA as follows:

Base 630-640 SspI
Base 760-870 NruI, StuI, XhoI, BglII, ClaI, SphI, NcoI, KpnI, SmaI, SstI, EcoRI, XbaI, HindIII, PstI, MluI, AccI, SalI, AatII, NdeI. BamHI. EcoRV, NaeI.
Base 1610-1619 SphI

Fusible ends were produced from the source DNA by ClaI and AccI for the fusion between base 546 and 547; by HpaI and BglII at for the fusion between base 1035 and 1036 and by HindIII and HincII for the fusion between base 1412 and 1413.

In SEQ ID No 2, the original nucleotide source DNA sequences have been changed by SDM at the following points:

Base 3	was C	now A	Creates EcoRI
Base 725-727	was GAT	now AAG	Removes ClaI
Base 768-770	was GTC	now ATT	Creates SspI
Base 1165-1167	was TAA	now GTT	Creates HpaI
Base 1281	was G	now C	Removes ClaI
Base 1356	was T	now A	Removes SspI
Base 1616	was G	now T	Removes AccI

Restriction endonuclease sites are provided in regions of DNA as follows:

Base 1-10 EcoRI
Base 180-190 XmnI
Base 760-770 SspI
Base 890-1000 NruI, StuI, XhoI, BglII, ClaI, SphI, NcoI, KpnI, SmaI, SstI, EcoRI, XbaI, HindIII, PstI, MluI, AccI, SalI, AatII, NdeI, BamHI, EcoRV, NaeI.
Base 1740-1751 SphI

Fusible ends were produced from the source DNA using HpaI and BglII for the fusion between 1167 and 1168; and using HindIII::HincII for the fusion between 1543 and 1544.

In SEQ ID No 3 derived from pVT100-U (bases 1-290 and 2295-3400) and pMTLJ (bases 291-2294), the original nucleotide source DNA sequences have been changed at the following points:

Base 425	was T	now C	Removes SspI

Restriction endonuclease sites are provided in regions of DNA as follows:

Base 1-10 SspI
Base 1360-1370 DraI
Base 2520-2530 HpaI

Fusable ends were produced from the source DNA using RsaI::EcoRV for the fusion between bases 290 and 291 and using EcoRV::RsaI for the fusion between bases 2294 and 2294.

In SEQ ID No 4 derived from pVT100-U (bases 1-290 and 1244-3249), pMTL4/CJ (bases 291-426 and 1214-2143) and pCM4 (bases 427-1213), the original nucleotide source sequences have ben changed at the following points:

Base 676	was A	now G	Removes EcoRI
Base 976	was C	now A	Removes NcoI
Base 985	was A	now G	Removes SspI

Restriction endonuclease sites are provided in regions of DNA as follows:

Base 1-10 SspI
Base 2370-2380 HpaI

Fusible ends were produced from the using RsaI::EcoRV for the fusion between bases 290 and 291, SspI::BamHI for the fusion between bases 426 and 427, BamHI::DraI for the fusion between bases 1213 and 1214 and EcoRV::RsaI for the fusion between bases 2143 and 2144.

In SEQ ID No 5 the original nucleotide source sequence has been changed at the following points:

Base 150	was C	now G	Removes NdeI
Base 289	was G	now C	Removes HpaI
Base 440	was C	now G	Removes NcoI
Base 563	was C	now T	Removes StuI
Base 1063	was G	now C	Removes AccI

Restriction endonuclease sites are thus absent from this sequence.

In SEQ ID No 6 the original nucleotide source sequence has been changed at the following points:

Base 294	was T	now C	Removes ClaI
Base 780	was C	now T	Removes EcoRI

Restriction endonuclease sites are provided in regions of DNA as follows:

Base 380-390 KpnI

Example 1: Expression Cassettes. An example nucleotide composition of the expression cassette containing the essential elements of this invention is designated SEQ ID 1, and was formed by fusing DNA regions from PGK (base 1-546 and base 1036-1411), REP2 (base 547-635), lacZ' (base 636-1035) and ADH1 (base 1412-1619); base numbers are those in SEQ ID No 1 not source DNA. Prior to fusion, the sequence composition of each element was altered to varying extents using site-directed mutagenesis (SDM). In the majority of cases the changes were made either to eliminate a restriction enzyme recognition common to the polylinker region within lacZ', or to create a restriction recognition site to facilitate the construction of the cassette. To compare the advantages of the novel promoter element, a second cassette was constructed, which contained no REP2 derived nucleotides, to act as a control. The sequence composition of this control cassette is shown as SEQ ID No 2.

The expression cassettes consist of the E. coli lacZ' gene, containing the pMTL23 polylinker cloning sites (Chambers et al., 1988), sandwiched by nucleotide signals for transcriptional initiation and termination. The transcriptional initiation signals of the hybrid promoter are provided by a unique combination of sequences derived from the promoters of PGK and REP2. The upstream activating sequence (UAS) element and TATA-box are from the PGK promoter and are fused to the 86 nucleotides residing immediately 5' to the 2 μm plasmid REP2 gene. The REP2 promoter is constitutive in nature (Som et al., 1988), and not generally regarded as a "strong" yeast promoter.

Within the hybrid promoter, the REP2 region is also responsible for

providing the expression cassette with promoter activity in E. coli. The region used contains sequence motifs which exactly correspond to those sequences necessary for transcription in procaryotes such as E. coli. Thus two hexanucleotide sequences are present, TTGACA and TATAAT, which exactly correspond to the consensus -35 and -10 boxes of E. coli promoters (Harley and Reynolds, 1987), and the spacing between them, 18 bp, is also consistent with a functional E. coli promoter. In addition, the AUG start codon of REP2 is preceded by the nucleotide motif -AGAA-.

The transcription initiation and termination signals flank unique restriction enzyme recognition sites into which heterologous genes may be inserted; with the exception of SspI, these sites form part of the lacZ' structural gene. Their location within the lacZ' gene allows the rapid detection of recombinant clones derived from the plasmid. The lacZ' gene encodes the alpha-peptide of β-galactosidase, such that when produced in E. coli hosts carrying the lacZ delta M15 mutation β-galactosidase leads to return of ability to metabolise the chromogenic substrate X-Gal and the production of blue colonies on agar medium supplemented with X-Gal. The insertion of heterologous DNA into the cloning sites of the expression cassette results in the inactivation of lacZ' and thus cells transformed with recombinant plasmid therefore produce colourless colonies on agar medium supplemented with X-Gal (Vieria and Messing, 1982).

The cassette is designed such that heterologous genes to be expressed are cloned using the SspI site and one of the recognition sites from within the polylinker. The SspI site (see list of sites in SEQ ID No 1 and 2 above) is located some 106 nucleotides 5' to the translational start of lacZ' and displacement of the DNA normally found between SspI and the polylinker within lacZ' results in recombinant plasmids which no longer confer a blue colouration on cells in the presence of X-Gal.

In the case of the PGK::REP2 promoter the ATA of the hexanucleotide sequence AATATT equates to the ATG start of the REP2 structural gene. In the case of the control expression cassette, the same triplet corresponds to the ATG start of the PGK structural gene. In both cases, when the cassettes are digested with SspI, the DNA is cleaved between the AT and A of the ATA triplet and a blunt-end is generated.

A DNA fragment carrying the gene to be expressed is then modified such that the first nucleotide of its blunt-ended, 5'-end is the "G" of the translational start codon of the structural gene. The 3'-end of this fragment may have any cohesive end compatible with those that can be generated by cleavage at the hexanucleotide recognition sites within lacZ'. Subsequent fusion of the 5' "G" nucleotide of the heterologous gene to the "AT" blunt-end of the cassette generated by SspI cleavage creates an ATG which is synonymous with both the translational start the heterologous gene and that of the structural genes from which the promoter elements were derived, ie., PGK in the case of the control cassette and REP2 in the case of the hybrid promoter.

The net result of the utilisation of this cloning strategy is that no changes are made to the nucleotides within the 5' untranslated region of the resultant mRNA, nor are any changes made to the codons of the gene being expressed. This would certainly not be true if a heterologous gene was merely inserted into the sites located solely in the polylinker region.

The method of choice used to allow the isolation of the heterologous gene as a blunt-ended fragment lacking the first two nucleotides of the translational start codon involves creating a recognition site for the restriction enzyme PstI at the start of the gene such that the terminal "G" of the created hexanucleotide sequence CTGCAG corresponds to the "G" of the genes translational start. The site created in the gene need not be PstI, but any site conforming to the consensus CNNNNG (where "N" is equivalent to, any nucleotide) which is cleaved by a restriction enzyme immediately before the "G" nucleotide to give a DNA terminus with a 3' overhang, ie., 3'-NNNN. Similarly, the recognition site used in the expression cassette need not be solely restricted to that of SspI, but can be any restriction site conforming to the consensus NATTAN (where "N" is equivalent to any nucleotide) which can be cleaved by a restriction enzyme between the two "T" nucleotides to give a blunt-end.

One potential problem with this cloning strategy occurs if the heterologous gene contains an internal PstI site. Two possible solutions are, firstly that the gene be inserted in a "two-step"

cloning strategy utilising another internal site 5' to the problem PstI site. Secondly, an oligonucleotide can be designed such that its 5' end corresponds to the G residue of the ATG translational start point. If this oligonucleotide is used in a PCR catalysed reaction to isolate the gene of interest, then cleavage with PstI is unnecessary. However, the original "PstI" strategy is preferable to this latter strategy, since PCR products have frequently been shown to have slightly heterogeneous termini (Hemsley et al., 1989).

Example 2: Preparation of Expression Vectors: A new series of vector backbones were constructed (see below) essentially being replication regions from the E. coli plasmid ColE1 and the yeast 2 μm plasmid. For selection in E.coli they carried either the bacterial cat or bla gene, conferring resistance to chloramphenicol (Cm) and ampicillin (Amp), respectively. The markers allowing selection in S. cerevisiae were either the LEU2 or URA3 gene, which convert appropriately deficient host strains to prototrophy. In the latter case, two alleles were constructed. Plasmids are shown in Fig 4.

Regardless of the nature of the selectable marker, of bacterial or yeast origin, every vector contains a unique SspI site between the bacterial selectable marker and the 2 μm replication origin. It was into this site that the expression cassette and control cassette were inserted. The former was isolated as a 1.6 kb XmnI/SspI fragment, and the latter as a 1.75 kb EcoRI/ SphI fragment. Both DNA fragments were blunt-ended by treatment with T4 DNA polymerase prior to their insertion into the SspI site. The orientation of insertion was such that lacZ' was counter transcribed relative to bla or cat.

Vector characteristics: CRM = chloramphenicol resistance marker. Gene markers transcribe away from STB but can transcribe toward it.

pMTL 8110: CRM, leu-dj gene marker, no cassette.

pMTL 8120: CRM, a defective S. cerevisiae URA3 gene and no cassette.

pMTL 8130: CRM, ura3-dj gene marker and no cassette.

pMTL 8131: CRM, ura3-dj gene marker and a cassette driven by the PGK promoter.

pMTL 8133: CRM, a defective S.cerevisiae URA3 gene and an expression cassette driven by the PGK:REP2 promoter.

pMTL 8140: CRM, leu2-dj gene marker and no cassette.

The vectors contain a minimum of 19 unique cloning sites in addition to the unique SspI sites. Non-unique sites are given in Table 2.

Evaluation of the Expression Cassettes: The capabilities of the expression system were initially assessed using the neo gene of the transposon Tn903. It encodes aminoglycoside-3'-phosphotransferase type I (APH1), which confers resistance to the antibiotic kanamycin and its analogue G418 (Haas and Dowding, 1975). The gene was available as a "Genblock (1.5 kb EcoRI fragment) from Pharmacia. This fragment was inserted into the EcoRI site of plasmid pUC8 to give plasmid pGENBLOCK. PCR was used to amplify a 1.11 kb fragment carrying the entire structural gene. During PCR the design of the oligonucleotide employed as the primer to the 5' end of the gene was such that a PstI recognition site was created. Specifically, the CAG of the created hexanucleotide sequence CTGCAG replaced the neo translational start codon.

The amplified fragment was digested with PstI and the overhanging 3' ends were removed by utilising the 3' to 5' exonuclease activity of T4 DNA polymerase. The fragment was then ligated with the pMTL 8111 and pMTL8113 expression vectors which had previously been digested with SspI and StuI and dephosphorylated. Colourless transformants were screened for the presence of the neo insert and the correct orientation by restriction analysis, and the plasmids obtained designated pKAN8111 and pKAN8113, respectively. Cells of S.cerevisiae strain AS33 carrying either plasmid were shown to be resistant to G418 at levels up to 3 mg/ml, indicative of extremely efficient expression of the neo gene. In contrast, only E. coli cells containing pKAN8113 were able to grow in the presence of G418 (at levels greater than 1 mg/ml). Lysates prepared from yeast carrying either plasmid cells were subjected to SDS-PAGE and the Comassie stained electro -phoretograms scanned with a Joyce-Loebell laser densitometer. A protein band equating to a size of 30,000 daltons was estimated to represent some 5% of the cell's soluble protein.

Primer Extension Analysis of S. cerevisiae mRNA: In order to ascertain the site(s) of transcriptional initiation within the two fusion promoters, mRNA was isolated from exponentially growing YEPD cultures of S.cerevisiae AS33 containing pKAN8111 and pKAN8113. A 25

bp oligonucleotide primer was synthesised, complementary to the coding strand at +53 to +77 within the neo coding region, and purified to homogeneity. It was not necessary to consider wild-type chromosomal transcription, since the neo gene does not occur chromosomally. Primer extension was performed and the products compared with end-labelled DNA sequence reactions primed with the same oligo -nucleotide primer.

The results demonstrated that the mRNA transcriptional start point (tsp) of the PGK promoter of pKAN8111 maps to nucleotide at -42. This is one nucleotide further from the AUG than that reported by Van den Heuvel et al. (1989) and 2 nucleotides further than that determined by Mellor et al. (1985). Over 90% of transcription from the PGK:REP2 promoter of pKAN8113 appeared to initiate at nt -87 at a G residue. Thus, REP2 promoter tsp site plays no role in transcription, rather factors within the PGK portion of the promoter direct the position and pattern of RNA initiation. Rathjen and Mellor (1990) have shown that initiation in PGK is reliant on two cis-acting sequences, the TATA element at nt -152 and a sequence, 5'-ACAGATCA-3', located immediately 5' to the site of RNA called the "determinator". In the PGK:REP2 promoter, however, the first "C" of the determinator has been deleted without any apparent effect.

Over Production of PAL in E. coli and S. cerevisiae: A PstI site was introduced over the authentic translational start point of a PAL cDNA clone from Rhodosporidium toruloides (Anson et al., 1986; Anson et al., 1987; Rasmussen and Orum, 1991) using PCR-mediated SDM (Higuchi et al..1988); an XbaI site lying 115 bp downstream from the PAL UAG termination codon. The PAL gene was excised as a PstI (blunt)/XbaI fragment and cloned into SspI/XbaI cut pMTL 8131 and pMTL 8133 to generate pPAL 8131 and pPAL 8133 respectively. The expression of PAL in S.cerevisiae strain AS33 is shown in Table 3. The lower expression levels obtained when cells are grown in rich selective media probably reflect a drop in plasmid copy number (Rose and Broach, 1990), although a decline in promoter activity and/or increase in mRNA turnover cannot be discounted.

The crude cell-free extracts were analysed by PAGE (Fig 5) and a band corresponding to a protein of approximate MW 75kD, which is present

only in the strain carrying pPAL 8133, was detected. This corresponds to the molecular weight of the PAL monomer. The gel was scanned with a laser densitometer (Joyce-Loebell) which calculated that this band constitutes approximately 9% of total soluble cell protein. This correlates well with the figure obtained by comparing the specific activity of purified PAL at 30°C with the assay data. This would indicate that the vast majority of the recombinant PAL is produced in an active form.

PAL expression levels in E.coli TG1 (Table 3) confirmed the finding that the PGK:REP2 promoter is highly active in E.coli, whilst the native PGK promoter is inactive. Deletion of part of the putative "-35" region resulted in partial loss of activity of this promoter in E.coli (data not shown), indicating that it is indeed these signals which are activating transcription in E.coli. Quantitative scanning of polyacrylamide gels indicated PAL expression levels to be of the order of 10% total soluble cell protein.

MATERIALS AND METHODS: A.1 Strains, Plasmids, Transformation and Media.

The S.cerevisiae strain AS33 (a, his3-11, his3-15, leu2-3, leu2-112, ura3-251, ura3-373, trp1) was used throughout. Yeast were transformed by electroporation (Becker and Guarente, 1991) and transformants selected by their ability to complement the appropriate auxotrophic allele. E.coli strain TG1 (Carter et al., 1985) was used as host for all DNA manipulations and bacterial expression studies. Plasmid pVT100-U (Vernet et al., 1987) was a kind gift from Dr. T. Vernet and plasmid pCM4 (Close and Rodriguez, 1982) obtained from Pharmacia. All DNA manipulations were carried out essentially as described in Sambrook et al. (1989). Polymerase chain reaction (PCR) was carried out on a programmable thermal cycler using Taq DNA polymerase (Amplitaq, Perkin-Elmer Cetus). DNA sequencing was based on the modified chain termination procedure described by Tabor and Richardson (1987). Oligos were synthesised using an Applied Biosystems 380A DNA synthesiser.

Site-directed mutagenesis (SDM) was performed by a number of techniques. Initially, mutants were created using a derivation of the method described by Carter et al., (1985). Subsequently, SDM was

performed by a method based on that described by Kunkel (1985).

Latter mutagenesis experiments were carried out using a novel coupled-primer method for SDM. Essentially, a PCR product was generated using kinased oligos, one of which contained the mutagenic mis-match, whilst the other was located at a point on the target plasmid such that a restriction site, which was unique in the plasmid, lay between the two primers. This PCR product was mixed with an equimolar amount of target plasmid DNA, which had been passaged through an E.coli dut ung strain, and linearised at the unique restriction site. The DNA mixture was denatured at 65°C for 5 min in denaturing buffer (0.2 M NaOH, 0.2 mM EDTA), before neutralisation (2 M NH_4Ac, pH 4.5) and subsequent ethanol precipitation. The DNA was redissolved in annealing buffer (20 mM Tris-HCl, pH 7.4; 2 mM $MgCl_2$, 50 mM NaCl) and annealed for 15 min at 37°C. Extension reactions were at 37°C for 1 hr in a buffer containing 1x TM buffer, 5 mM DTT, 500 µM dNTPs, 250 µM rATP, 2 units T4 DNA ligase and 10 units Sequenase. Aliquots of this reaction were then transformed into E.coli TG1.

Typical mutagenesis frequencies were in the region of 30%. This technique obviates the need for sub-cloning into specialised vectors or the use of repair-deficient strains. Assay for PAL Activity: PAL levels in cell-free extracts were assayed by the method of Abell and Shen (1987). The production of cinnamic acid can be monitored spectrophotometrically at 290 nm. 0.67 ml distilled water, 0.17 ml 6x assay buffer (500 mM Tris-HCl pH 8.5) and 0.17 ml L-phenylalanine (50 mM in 100 mM Tris-HCl pH 8.5) were combined in a 1 ml cuvette (Hughes and Hughes Ltd., UV range). The cuvette and its contents were pre-warmed to 30°C and placed in a Perkin-Elmer Lambda 2 Spectrophotometer. 25 µl of crude cell extract was added and the absorbance at 290 nm was monitored for 30 seconds at 30°C.

One unit of enzyme was defined as the amount catalysing formation of 1 µmol cinnamic acid per minute under the assay conditions used. The molar absorption coefficient for cinnimate at 290 nm, 30°C, pH 8.5 (E_{290}) was taken as 9×10^3 litre/mol/cm (Abell and Shen, 1987). The level of PAL activity can then be calculated as follows:

$$IU/ml = \frac{deltaA290 \times 10^3 \times Dilution\ Factor}{E\ 290 \times \mu l\ of\ sample / 1000}$$

Protein concentrations were determined by the method of Bradford (1976).

Derivation of the Expression Cassette: The initial stages involved in construction were common to each cassette. Two mutagenic oligonucleotides were employed to PCR amplify a 410 bp fragment of pMTL23 encompassing lacZ' and the lac po region (Chambers et al., 1988). The resultant modified fragment possessed a SspI site at position -106 (relative to the lacZ' translational start codon) and a HpaI site at nucleotide position +293 (relative to the lacZ' start codon). The transcriptional termination signals of the PGK were cloned from S. cerevisiae strain LL20 chromosomal DNA as a 373 bp BglII/ HindIII fragment into M13mtl20 (Chambers et al., 1988). The restriction enzyme recognition sites for ClaI and SspI were eliminated by SDM, and the DNA reisolated as a BglII/ HindIII fragment. The 3' end of the ADH1 locus was sub-cloned from pVT100-U (Vernet et al., 1987) as a 335 bp SphI/HindIII fragment into similarly cleaved M13mp18. An AccI recognition site removed by SDM, and the region carrying the desired transcriptional termination signals reisolated as a 206 bp HincIII/SphI fragment. The three DNA fragments specifying lacZ', the PGK transcriptional terminator and the ADH1 transcriptional terminator were then fused, by ligation with DNA ligase, in the order and orientation shown in SEQ ID No 1 and 2. Prior to fusion, the staggered ends of the DNA fragment encompassing the PGK transcriptional terminator (those generated by cleavage with BglII and HindIII) were blunt-ended by treatment with T4 DNA polymerase.

To complete the control cassette, a 3.1 kb HindIII fragment carrying the PGK gene of S. cerevisiae strain LL20 was inserted into M13mp8 and SDM employed to create restriction recognition sites for EcoRI and SspI. In the case of the SspI recognition site, its position was such that the ATG triplet corresponding to the translational start codon of the PGK structural gene became the ATA of the SspI site, AATATT. A 766 bp fragment encompassing the transcriptional signals of PGK was then isolated from the resultant mutagenic M13 clone, M13PGK-J, following cleavage with EcoRI and SspI, and ligated to the

999 bp SspI/ SphI fragment composed of lacZ'::PGK::ADH1, such that the SspI recognition site was retained.

To complete the expression cassette containing the hybrid promoter, a 1.8 kb HindIII fragment (nucleotides 4621 to 92 of the sequence of Hartley and Donelson, 1980) carrying the promoter of the 2 μm plasmid REP2 gene was subcloned into the equivalent site of M13mp8. Recognition sites for the restriction enzymes AccI and SspI were then created in the sequence by SDM. This was achieved by changing the hexanucleotide sequences GTTGTT and AATGGA (respective nucleotide positions 5288 to 5283 and 5199 to 5194; Hartley and Donelson, 1980) to GTCGAC and AATATT, respectively. Additionally, two "G" nucleotides (positions 557 and 580 in SEQ ID No 2) were both changed to "T". The recombinant plasmid obtained was designated M13REP2-J. An additional recognition site for the restriction enzyme ClaI was also created within the PGK derived region of M13PGK-J. The changes made are detailed above in the section on features of SEQ ID No 2, at positions 725 and 727. The transcriptional signals of PGK were then isolated as a 540 bp XmnI/ ClaI fragment, and ligated to a 90 bp AccI/ SspI fragment isolated from M13REP2-J, such that fusion occurred between the compatible ClaI and AccI derived DNA sticky ends. The resultant 630 bp fragment was then ligated to the 999 kb SspI/ SphI fragment composed of lacZ'::PGK::ADH1, such that the SspI recognition site was retained.

Nucleotide sequence analysis of the various components of the constructed cassettes indicated the presence of nucleotide differences to previously published sequences, presumably a consequence of strain variation. Specifically, several base differences were observed between the transcriptional initiation and termination regions of the PGK gene used here and that determined by Hitzeman et al. (1982). By reference to SEQ ID No 2, the Hitzeman et al. (1982) sequence has 5 "A" nucleotides rather than the 4 beginning at position 760, lacks the "G" at position 729, has an extra "A" between nucleotides 1399 and 1400, and an extra "T" nucleotides between position 1493 and 1494. Similarly, the "A" nucleotide at position 1663 was found to be a "G" in the ADH1 gene determined by Bennetzen and Hall (1985).

Two additional nucleotide mutations occurred during the construction

of the expression cassette containing the hybrid promoter, around the junction point between the PGK promoter and the REP2 leader region. Thus, a "C" nucleotide base has been deleted from between positions 538 and 539 in SEQ ID No 1 (the "C" at position 716 in SEQ ID No 2), and the nucleotide base at position 543 has become an "A", rather than the "C" found in the equivalent position of the strain LL20 PGK promoter (position 721 of SEQ ID No 2).

Example 3: Derivation of E. coli/ S. cerevisiae Shuttle Vectors: Provision of E. coli maintenance and replication functions: the first stage in the construction of the new E.coli/S.cerevisiae vectors was to combine the replicative functions of an E. coli plasmid with that of a S. cerevisiae plasmid. Two basic vectors were made, pMTL8000 and pMTL8100. As shown in Figure 6, both were constructed by isolating a 1.4 kb RsaI, which encompassed the origin of replication and STB locus of the 2 μm plasmid, from plasmid pVT100-U (Vernet et al., 1987), and inserting it into the unique EcoRV sites of either pMTLJ or pMTLCJ to give pMTL8000 or pMTL8100, respectively.

Plasmid pMTLJ was derived from pMTL4 (Chambers et al., 1988), by eliminating the recognition site for the restriction enzyme SspI using the plasmid SDM method. The steps involved in the derivation of pMTLCJ are shown in Figure 7. Essentially, a 0.8 kb BamHI fragment, encoding cat, was excised from plasmid pCM4 (Close and Rodriguez, 1982) and inserted into the BamHI site of M13mp8. The ssDNA prepared from the resultant recombinant was then used as a template in successive SDM experiments to eliminate restriction enzyme recognition sites for EcoRI, NcoI and SspI from the cat structural gene. ds DNA of the mutated M13 recombinant was then prepared, the modified cat gene excised as a 0.8 kb BamHI fragment, blunt-ended by treatment with DNA polymerase I Klenow fragment and ligated to a 1.1 kb SspI/DraI fragment encompassing the replication region of plasmid pMTL4 to give pMTLCJ.

The nucleotide sequences of pMTL8000 and pMTL8100 are shown as SEQ ID No 3 and 4. The 2 μm replication region resides between nucleotides 3154 to 3376 of pMTL8000 and 3003 to 3225 of pMTL8100. The STB locus is between nucleotides 2526 to 2817 of pMTL8000 and between 2375 and 2666 of pMTL8100. The bla structural gene begins at nucleotide 444 of

pMTL8000 and ends at position 1304. The cat structural gene of pMTL8100 begins at nucleotide 461 and ends at position 1117. In both cases, the amino acid sequence of the encoded proteins are shown below the first nucleotide of the corresponding codon in the single letter code. The ColE1 origin of replication lies at nucleotides 2063-2068 and 1912-1917 in pMTL8000 and pMTL8100, respectively.

Provision of markers for plasmid selection in S. cerevisiae: The basic backbone of the vector series was completed by inserting DNA sequence elements into pMTL8000 and pMTL8100 which allowed direct selection of the described plasmid series in appropriate auxotrophic S.cerevisiae host strains. Two different selective markers were employed.

Firstly, a 1.17 kb BglII fragment containing the S.cerevisiae URA3 gene was sub-cloned from pVT100-U into the BamHI site of M13mp8. The ssDNA prepared from the resultant recombinant was then used as a template in successive SDM experiments designed to eliminate unique restriction enzyme recognition sites for NdeI, NcoI, and StuI, and two AccI restriction sites. This modified gene was designated the URA3-J allele. The complete sequence of the DNA fragment actually inserted into the eventual expression vectors (see below) is shown as SEQ ID No 5. The URA3 structural gene initiates at nucleotide 234 and terminates at nucleotide 1034. The amino acid sequence of the encoded protein is shown in the single letter code below the first nucleotide of the corresponding codon.

In addition to the standard URA3 selectable marker, a promoterless version, ura3-d was also created. SDM was employed to create a HpaI site at nt -47 (relative to the AUG start codon) in the URA3-J allele. This equates to changing the "C" nucleotide at position 189 to a "G". Subsequent excision of the gene by cleaving with HpaI at this point removes all sequences necessary for activation of the URA3 gene (Roy et al., 1990), whilst retaining the major transcriptional start points at nt -38 and -33 (Rose and Botstein, 1983). It was anticipated that plasmids endowed with ura3-d would possess elevated plasmid copy number under selective conditions, as observed with plasmids carrying an equivalent promoterless LEU2 gene, leu2-d (Ecrhart and Hollenberg, 1983).

The second selectable marker used was the LEU2 gene. This was sub-cloned as a 1.46 kb SspI fragment from pMA300 (Montiel et al., 1984) into the SmaI site of pUC8. This fragment lacks the sequences mapped as the UAS of LEU2 at -201 to -187 (Tu and Casadaban, 1990), and disrupts the sequence upstream from LEU2 which codes for a putative regulatory peptide (Andreadis et al., 1982). However, it retains the TATA-like AT-rich sequence between bases -118 to -111 that has been proposed as a site for the yeast TATA-binding factor TFIID (Tu and Casadaban, 1990). The recombinant, pUC8-derived plasmid carrying LEU2 was used as a template in SDM experiments to remove the recognition sites for the restriction enzymes ClaI and EcoRI. In the sequence shown as SEQ ID No 5 the URA3 structural gene initiates at nucleotide 234 and terminates at nucleotide 1034. The amino acid sequence of the encoded protein is shown in the single letter code below the first nucleotide of the corresponding codon.

To insert the three alleles URA3-J, ura3-dJ and leu2-dJ into the unique pMTL 8000 and pMTL 8100, each allele was excised from the appropriate plasmid and converted, where necessary, to a blunt-ended DNA fragment. In the case of URA3-J, plasmid pURA3-J was cleaved with AccI (cleaving at a site within the pUC8 polylinker region) and SmaI (cleaving at a SmaI site residing some 79 nucleotides 3' to the translational stop codon of URA3) and the released c. 1.1 kb fragment carrying URA3 treated with T4 DNA polymerase. The exact sequence of the blunt-ended fragment generated is shown in SEQ ID No 5. A c.0.92 kb blunt-ended fragment carrying the ura3-dJ allele was obtained by cleaving plasmid pURA3-dJ with HpaI and SmaI.

The nucleotide sequence of the fragment obtained exactly corresponds to the sequence shown in SEQ ID No 5 between nucleotide 192 and 1115, inclusive. Plasmid pLEU2-dJ was cleaved with EcoRI (at the recognition site within the pUC8 polylinker region) and AccI (at a recognition site located 100 nucleotides 3' to the translational stop of URA3. The exact sequence of the blunt-ended fragment generated is shown in SEQ ID No 6.

All three isolated fragments carrying URA3-J, ura3-dJ and leu2-dJ were inserted into the unique HpaI site of both pMTL8000 and pMTL8100. With one exception, all the recombinant plasmids obtained no longer

contained HpaI sites. The exceptions were the pMTL8000 and pMTL8100 derivatives carrying ura3-dJ, where the HpaI site is retained at the junction point lying 5' end to the gene. To avoid compromising the segregational stability of the plasmids by potential read-through from the selective markers into STB (Murray and Cesareni, 1986), clones were orientated such that the yeast selective markers transcribed away from the STB locus. For comparative purposes, a plasmid containing the leu2-dJ allele transcribing towards STB were also constructed.

Physical Characterisation of Constructed Vectors: Before proceeding to insert the expression cassette into the vector series, the basic backbone vectors were assessed with regard to their stability (segregational and structural) and copy number.

Measurement of plasmid segregational stability in S. cerevisiae: Plasmid segregational stability was estimated using methodology described by Spalding and Tuite (1989). This involved following the loss of a plasmid-encoded phenotypic marker over a number of generations under non-selective conditions. The results are presented in Table 1. All plasmids exhibited a greater degree of segregational stability than that of the well characterised S.cerevisiae cloning vector YEp24 (Botstein et al., 1979).

Measurement of structural stability: The structural stability of plasmids in S. cerevisiae was assessed by transforming each plasmid into strain AS33, growing cells for approximately 30 generations under selective conditions, and then transferring each plasmid back to E. coli by the procedure of Hoffman and Winston (1987). Plasmid DNA was then prepared, by the method of Holmes and Quigley (1981), from the resultant E. coli transformants and subjected to restriction enzyme analysis. The restriction patterns obtained with all such plasmids isolated from E. coli, using the enzymes SspI and EcoRV, was identical to that of the CsCl-purified DNA originally transformed into strain AS33.

Estimation of plasmid copy number: Plasmid copy number determination was based on the non-isotopic technique of Futcher and Cox (1984). Approximately 5 µg of total yeast DNA was digested simultaneously with EcoRI and EcoRV. Following agarose gel electrophoresis, a negative

image of the restriction "spectrum" was scanned using a laser densitometer (Joyce-Loebell). The intensity of the band corresponding to plasmid DNA was compared with that of the 2.8 kb rDNA EcoRI fragment. The rDNA was assumed to be present at 140 tandem copies (Philipssen et al., 1991). Plasmid copy number was then calculated as follows:

$$\text{Plasmid Copy Number} = \frac{\text{Area under plasmid peak}}{\text{Area under rDNA 2.8kb peak}} \times \frac{2.8}{\text{plasmid size (kb)}} \times 140$$

Using this method, the copy numbers of the basic plasmid vectors in S.cerevisiae were compared to previously characterised high copy number (pMA3a; Spalding and Tuite, 1989) and low copy number (YEp24; Botstein et al., 1979) plasmids. The results in Table 1 confirm that low copy number (pMTL 8120) and high copy number (pMTL 8110, 8130 and 8140) versions, of the vectors described in the present invention, have been constructed.

Table 1 Segregational stability and copy number analysis of the pMTL 81X0 series of vectors.

Plasmid	Cells contg. * Plasmid (%)	Plasmid loss/ cell div(10^{-2})	Average copy number/cell
pMTL 8110	84.5	0.842	111
pMTL 8120	77.5	1.174	50
pMTL 8130	82.0	0.992	151
pMTL 8140	85.5	0.783	106
YEp24(URA3)	76.0	1.372	48
pMA3a(leu2-d)	ND	ND	106

* After 20 generations of non-selective exponential growth.

Segregational stability was performed using methodology described by Spalding and Tuite (1989) and is an average of two or more independent experiments. Copy number data is for cells grown in minimal media and is based on the assumption that all cells contain plasmid under these conditions. The selective marker present within each vector is shown in brackets. R = reverse orientation. ND = not determined.

Table 2 Non-unique restriction sites present within the polylinkers of the pMTL 8XXX series of vectors.

Marker	PGK	No Promoter	PGK:REP2
leu2-d	EcoRV.Kpn1.Sst1	EcoRV.Kpn1	EcoRV.Kpn1.Sst1
URA3	EcoRV	EcoRV	EcoRV
ura3-d	EcoRV.Sst1	EcoRV	EcoRV.Sst1
leu2-d	EcoRV.Kpn1.Sst1	EcoRV.Kpn1	EcoRV.Kpn1.Sst1

Table 3 Expression of PAL in S.cerevisiae AS33 and E.coli TG1. Figures refer to units $x10^{-2}$/mg soluble protein. At least three separate assays were performed for each sample and the maximum error range is indicated. ND = not determined. PAL = presence of PAL gene.

Strain and growth phase	pMTL 8130	pPAL 8133	pPAL 8131
S.cerevis'AS33 Minimal media Stationary	0	35.5 ± 2	18.1 ± 2
S.cerevis'AS33 YEPD Early exponent'	0	37.8 ± 3	ND
S.cerevis'AS33 YEPD Stationary	0	16.5 ± 1	8.5 ± 0.7
E.coliTG1 2xYT Stationary	0	35.2 ± 2	0

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SEQUENCE LISTING

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(D) STATE: OXFORDSHIRE
(E) COUNTRY: UNITED KINGDOM (GB)
(F) POSTAL CODE (ZIP): OX3 OTX

(ii) TITLE OF INVENTION: BIFUNCTIONAL EXPRESSION VECTOR
(iii) NUMBER OF SEQUENCES: 6

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release @1.0, Version @1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1619 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Saccharomyces cerevisiae
(ix) FEATURE:
(A) NAME/KEY: misc_recomb
(B) LOCATION: 546..547
(ix) FEATURE:
(A) NAME/KEY: misc_recomb

(B) LOCATION: 635..636
(ix) FEATURE:
(A) NAME/KEY: misc_recomb
(B) LOCATION: 1035..1036
(ix) FEATURE:
(A) NAME/KEY: misc_recomb
(B) LOCATION: 1411..1412
(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 550..555
(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 574..579
(ix) FEATURE:
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(B) LOCATION: 668..673
(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 692..697

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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AACTTACATT TACATATATA TAAACTTGCA TAAATTGGTC AATGCAAGAA ATACATATTT 480

GGTCTTTTCT AATTCGTAGT TTTTCAAGTT CTTAGATGCT TTCTTTTTCT CTTTTTTAAG 540

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TCAAACGAAC AATACTGGTA AAAGAAAACC AAAATATTAG TTAGCTCACT CATTAGGCAC 660

CCCAGGCTTT ACACTTTATG CTTCCGGCTC GTATGTTGTG TGGAATTGTG AGCGGATAAC 720

AATTTCACAC AGGAAACAGC TATGACCATG ATTACGCCAA GCTCGCGAGG CCTCGAGATC 780

TATCGATGCA TGCCATGGTA CCCGGGAGCT CGAATTCTAG AAGCTTCTGC AGACGCGTCG 840

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TGGGAAAACC CTGGCGTTAC CCAACTTAAT CGCCTTGCAG CACATCCCCC TTTCGCCAGC 960

TGGCGTAATA GCGAAGAGGC CCGCACCGAT CGCCCTTCCC AACAGTTGCG TAGCCTGAAT 1020

GGCGAATGGC GCGTTGATCT CCCATGTCTC TACTGGTGGT GGTGCTTCTT TGGAATTATT 1080

GGAAGGTAAG GAATTGCCAG GTGTTGCTTT CTTATCCGAA AAGAAATAAA TTGAATTGAA 1140

TTGAAATCCA TAGATCAATT TTTTTCTTTT CTCTTTCCCC ATCCTTTACG CTAAAATAAT 1200

AGTTTATTTT ATTTTTTGAA TATATTTTAT TTATATACGT ATATATAGAC TATTATTTAC 1260

TTTTAATGAT TATTAAGATT TTTATTAAAA AAAAATTCGT CCCTCTTTTT AATGCCTTTT 1320

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ATTTATGATT TTTATTATTA AATAAGTTAT AAAAAAAATA AGTTTATACA AATTTTAAAG 1500

TGACTCTTAG GTTTTAAAAC GAAAATTCTT ATTCTTGAGT AACTCTTTCC TGTAGGTCAG 1560

GTTGCTTTCT CAGGTATAGC ATGAGGTCGC TCTTATTGAC CACACCTCTA CCGGCATGC 1619

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1754 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Saccharomyces cerevisiae
(ix) FEATURE:
(A) NAME/KEY: misc_recomb
(B) LOCATION: 546..547
(ix) FEATURE:
(A) NAME/KEY: misc_recomb
(B) LOCATION: 635..636
(ix) FEATURE:
(A) NAME/KEY: misc_recomb
(B) LOCATION: 1035..1036
(ix) FEATURE:
(A) NAME/KEY: misc_recomb
(B) LOCATION: 1411..1412

(xi) SEQUENCE DESCRIPTION: SEQ ID NO 2:

GAATTCAACT CAAGACGCAC AGATATTATA ACATCTGCAT AATAGGCATT TGCAAGAATT 60

ACTCGTGAGT AAGGAAAGAG TGAGGAACTA TCGCATACCT GCATTTAAAG ATGCCGATTT 120

GGGCGCGAAT CCTTTATTTT GGCTTCACCC TCATACTATT ATCAGGGCCA GAAAAAGGAA 180

GTGTTTCCCT CCTTCTTGAA TTGATGTTAC CCTCATAAAG CACGTGGCCT CTTATCGAGA 240

AAGAAATTAC CGTCGCTCGT GATTTGTTTG CAAAAAGAAC AAAACTGAAA AAACCCAGAC 300

ACGCTCGACT TCCTGTCTTC CTATTGATTG CAGCTTCCAA TTTCGTCACA CAACAAGGTC 360

CTAGCGACGG CTCACAGGTT TTGTAACAAG CAATCGAAGG TTCTGGAATG GCGGGAAAGG 420

GTTTAGTACC ACATGCTATG ATGCCCACTG TGATCTCCAG AGCAAAGTTC GTTCGATCGT 480

ACTGTTACTC TCTCTCTTTC AAACAGAATT GTCCGAATCG TGTGACAACA ACAGCCTGTT 540

CTCACACACT CTTTTCTTCT AACCAAGGGG GTGGTTTAGT TTAGTAGAAC CTCGTGAAAC 600

TTACATTTAC ATATATATAA ACTTGCATAA ATTGGTCAAT GCAAGAAATA CATATTTGGT 660

CTTTTCTAAT TCGTAGTTTT TCAAGTTCTT AGATGCTTTC TTTTTCTCTT TTTTACAGAT 720

CATCAAGGGA AGTAATTATC TACTTTTTAC AACAAATATA AAACAATATT AGTTAGCTCA 780

CTCATTAGGC ACCCCAGGCT TTACACTTTA TGCTTCCGGC TCGTATGTTG TGTGGAATTG 840

TGAGCGGATA ACAATTTCAC ACAGGAAACA GCTATGACCA TGATTACGCC AAGCTCGCGA 900

GGCCTCGAGA TCTATCGATG CATGCCATGG TACCCGGGAG CTCGAATTCT AGAAGCTTCT 960

GCAGACGCGT CGACGTCATA TGGATCCGAT ATCGCCGGCA ATTCACTGGC CGTCGTTTTA 1020

CAACGTCGTG ACTGGGAAAA CCCTGGCGTT ACCCAACTTA ATCGCCTTGC AGCACATCCC 1080

CCTTTCGCCA GCTGGCGTAA TAGCGAAGAG GCCCGCACCG ATCGCCCTTC CCAACAGTTG 1140

CGTAGCCTGA ATGGCGAATG GCGCGTTGAT CTCCCATGTC TCTACTGGTG GTGGTGCTTC 1200

TTTGGAATTA TTGGAAGGTA AGGAATTGCC AGGTGTTGCT TTCTTATCCG AAAAGAAATA 1260

AATTGAATTG AATTGAAATC CATAGATCAA TTTTTTTCTT TTCTCTTTCC CCATCCTTTA 1320

CGCTAAAATA ATAGTTTATT TTATTTTTTG AATATATTTT ATTTATATAC GTATATATAG 1380

ACTATTATTT ACTTTTAATG ATTATTAAGA TTTTTATTAA AAAAAAATTC GTCCCTCTTT 1440

TTAATGCCTT TTATGCAGTT TTTTTTTCCC ATTCGATATT TCTATGTTCG GGTTCAGCGT 1500

ATTTTAAGTT TAATAACTCG AAAATTCTGC GTTCGTTAAA GCTGACACTT CTAAATAAGC 1560

GAATTTCTTA TGATTTATGA TTTTTATTAT TAAATAAGTT ATAAAAAAAA TAAGTTTATA 1620

CAAATTTTAA AGTGACTCTT AGGTTTTAAA ACGAAAATTC TTATTCTTGA GTAACTCCTC 1680

TTTCCTGTAG GTCAGGTTGC TTTCTCAGGT ATAGCATGAG GTCGCTCTTA TTGACCACAC 1740

CTCTACCGGC ATGC

2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3400 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Saccharomyces cerevisiae
(ix) FEATURE:
(A) NAME/KEY: misc_recomb
(B) LOCATION: 290..291
(ix) FEATURE:
(A) NAME/KEY: misc_recomb
(B) LOCATION: 2294..2295

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

AATATTTTAG TAGCTCGTTA CAGTCCGGTG CGTTTTTGGT TTTTTGAAAG TGCGTCTTCA 60

GAGCGCTTTT GGTTTTCAAA AGCGCTCTGA AGTTCCTATA CTTTCTAGCT AGAGAATAGG 120

AACTTCGGAA TAGGAACTTC AAAGCGTTTC CGAAAACGAG CGCTTCCGAA AATGCAACGC 180

GAGCTGCGCA CATACAGCTC ACTGTTCACG TCGCACCTAT ATCTGCGTGT TGCCTGTATA 240

TATATATACA TGAGAAGAAC GGCATAGTGC GTGTTTATGC TTAAATGCGT ATCCCGCAAG 300

AGGCCCGGCA GTCAGGTGGC ACTTTTCGGG GAAATGTGCG CGGAACCCCT ATTTGTTTAT 360

TTTTCTAAAT ACATTCAAAT ATGTATCCGC TCATGAGACA ATAACCCTGA TAAATGCTTC 420

ATTACTATTG AAAAAGGAAG AGTATGAGTA TTCAACATTT CCGTGTCGCC CTTATTCCCT 480

TTTTTGCGGC ATTTTGCCTT CCTGTTTTTG CTCACCCAGA AACGCTGGTG AAAGTAAAAG 540

ATGCTGAAGA TCAGTTGGGT GCACGAGTGG GTTACATCGA ACTGGATCTC AACAGCGGTA 600

AGATCCTTGA GAGTTTTCGC CCCGAAGAAC GTTTTCCAAT GATGAGCACT TTTAAAGTTC 660

TGCTATGTGG CGCGGTATTA TCCCGTATTG ACGCCGGGCA AGAGCAACTC GGTCGCCGCA 720

TACACTATTC TCAGAATGAC TTGGTTGAGT ACTCACCAGT CACAGAAAAG CATCTTACGG 780

ATGGCATGAC AGTAAGAGAA TTATGCAGTG CTGCCATAAC CATGAGTGAT AACACTGCGG 840

CCAACTTACT TCTGACAACG ATCGGAGGAC CGAAGGAGCT AACCGCTTTT TTGCACAACA 900

TGGGGGATCA TGTAACTCGC CTTGATCGTT GGGAACCGGA GCTGAATGAA GCCATACCAA 960

ACGACGAGCG TGACACCACG ATGCCTGTAG CAATGGCAAC AACGTTGCGC AAACTATTAA 1020

CTGGCGAACT ACTTACTCTA GCTTCCCGGC AACAATTAAT AGACTGGATG GAGGCGGATA 1080

AAGTTGCAGG ACCACTTCTG CGCTCGGCCC TTCCGGCTGG CTGGTTTATT GCTGATAAAT 1140

CTGGAGCCGG TGAGCGTGGG TCTCGCGGTA TCATTGCAGC ACTGGGGCCA GATGGTAAGC 1200

CCTCCCGTAT CGTAGTTATC TACACGACGG GGAGTCAGGC AACTATGGAT GAACGAAATA 1260

GACAGATCGC TGAGATAGGT GCCTCACTGA TTAAGCATTG GTAACTGTCA GACCAAGTTT 1320

ACTCATATAT ACTTTAGATT GATTTAAAAC TTCATTTTTA ATTTAAAAGG ATCTAGGTGA 1380

AGATCCTTTT TGATAATCTC ATGACCAAAA TCCCTTAACG TGAGTTTTCG TTCCACTGAG 1440

CGTCAGACCC CGTAGAAAAG ATCAAAGGAT CTTCTTGAGA TCCTTTTTTT CTGCGCGTAA 1500

TCTGCTGCTT GCAAACAAAA AAACCACCGC TACCAGCGGT GGTTTGTTTG CCGGATCAAG 1560

AGCTACCAAC TCTTTTTCCG AAGGTAACTG GCTTCAGCAG AGCGCAGATA CCAAATACTG 1620

TTCTTCTAGT GTAGCCGTAG TTAGGCCACC ACTTCAAGAA CTCTGTAGCA CCGCCTACAT 1680

ACCTCGCTCT GCTAATCCTG TTACCAGTGG CTGCTGCCAG TGGCGATAAG TCGTGTCTTA 1740

CCGGGTTGGA CTCAAGACGA TAGTTACCGG ATAAGGCGCA GCGGTCGGGC TGAACGGGGG 1800

GTTCGTGCAC ACAGCCCAGC TTGGAGCGAA CGACCTACAC CGAACTGAGA TACCTACAGC 1860

GTGAGCATTG AGAAAGCGCC ACGCTTCCCG AAGGGAGAAA GGCGGACAGG TATCCGGTAA 1920

GCGGCAGGGT CGGAACAGGA GAGCGCACGA GGGAGCTTCC AGGGGGAAAC GCCTGGTATC 1980

TTTATAGTCC TGTCGGGTTT CGCCACCTCT GACTTGAGCG TCGATTTTTG TGATGCTCGT 2040

CAGGGGGGCG GAGCCTATGG AAAAACGCCA GCAACGCGGC CTTTTTACGG TTCCTGGCCT 2100

TTTGCTGGCC TTTTGCTCAC ATGTTCTTTC CTGCGTTATC CCCTGATTCT GTGGATAACC 2160

GTATTACCGC CTTTGAGTGA GCTGATACCG CTCGCCGCAG CCGAACGACC GAGCGCAGCG 2220

AGTCAGTGAG CGAGGAAGCG GAAGAGCGCT AGCAGCACGC CATAGTGACT GGCGATGCTG 2280

TCGGAATGGA CGATACTTGT TACCCATCAT TGAATTTTGA ACATCCGAAC CTGGGAGTTT 2340

TCCCTGAAAC AGATAGTATA TTTGAACCTG TATAATAATA TATAGTCTAG CGCTTTACGG 2400

AAGACAATGT ATGTATTTCG GTTCCTGGAG AAACTATTGC ATCTATTGCA TAGGTAATCT 2460

TGCACGTCGC ATCCCCGGTT CATTTTCTGC GTTTCCATCT TGCACTTCAA TAGCATATCT 2520

TTGTTAACGA AGCATCTGTG CTTCATTTTG TAGAACAAAA ATGCAACGCG AGAGCGCTAA 2580

TTTTTCAAAC AAAGAATCTG AGCTGCATTT TTACAGAACA GAAATGCAAC GCGAAAGCGC 2640

TATTTTACCA ACGAAGAATC TGTGCTTCAT TTTTGTAAAA CAAAAATGCA ACGCGAGAGC 2700

GCTAATTTTT CAAACAAAGA ATCTGAGCTG CATTTTTACA GAACAGAAAT GCAACGCGAG 2760

AGCGCTATTT TACCAACAAA GAATCTATAC TTCTTTTTTG TTCTACAAAA ATGCATCCCG 2820

AGAGCGCTAT TTTTCTAACA AAGCATCTTA GATTACTTTT TTTCTCCTTT GTGCGCTCTA 2880

TAATGCAGTC TCTTGATAAC TTTTTGCACT GTAGGTCCGT TAAGGTTAGA AGAAGGCTAC 2940

TTTGGTGTCT ATTTTCTCTT CCATAAAAAA AGCCTGACTC CACTTCCCGC GTTTACTGAT 3000

TACTAGCGAA GCTGCGGGTG CATTTTTTCA AGATAAAGGC ATCCCCGATT ATATTCTATA 3060

CCGATGTGGA TTGCGCATAC TTTGTGAACA GAAAGTGATA GCGTTGATGA TTCTTCATTG 3120

GTCAGAAAAT TATGAACGGT TTCTTCTATT TTGTCTCTAT ATACTACGTA TAGGAAATGT 3180

TTACATTTTC GTATTGTTTT CGATTCACTC TATGAATAGT TCTTACTACA ATTTTTTTGT 3240

CTAAAGAGTA ATACTAGAGA TAAACATAAA AAATGTAGAG GTCGAGTTTA GATGCAAGTT 3300

CAAGGAGCGA AAGGTGGATG GGTAGGTTAT ATAGGGATAT AGCACAGAGA TATATAGCAA 3360

AGAGATACTT TTGAGCAATG TTTGTGGAAG CGGTATTCGC 3400

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3249 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Saccharomyces cerevisiae
(ix) FEATURE:
(A) NAME/KEY: misc_recomb
(B) LOCATION: 290..291
(ix) FEATURE:
(A) NAME/KEY: misc_recomb
(B) LoCATION: 426..42/
(ix) FEATURE:
(A) NAME/KEY: misc_recomb
(B) LOCATION: 1213..1214
(ix) FEATURE:
(A) NAME/KEY: misc_recomb
(B) LOCATION: 2143..2144

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AATATTTTAG TAGCTCGTTA CAGTCCGGTG CGTTTTTGGT TTTTTGAAAG TGCGTCTTCA 60

GAGCGCTTTT GGTTTTCAAA AGCGCTCTGA AGTTCCTATA CTTTCTAGCT AGAGAATAGG 120

AACTTCGGAA TAGGAACTTC AAAGCGTTTC CGAAAACGAG CGCTTCCGAA AATGCAACGC 180

GAGCTGCGCA CATACAGCTC ACTGTTCACG TCGCACCTAT ATCTGCGTGT TGCCTGTATA 240

TATATATACA TGAGAAGAAC GGCATAGTGC GTGTTTATGC TTAAATGCGT ATCCCGCAAG 300

AGGCCCGGCA GTCAGGTGGC ACTTTTCGGG GAAATGTGCG CGGAACCCCT ATTTGTTTAT 360

TTTTCTAAAT ACATTCAAAT ATGTATCCGC TCATGAGACA ATAACCCTGA TAAATGCTTC 420

AATAATGATC CACGAGATTT CAGGAGCTAA GGAAGCTAAA ATGGAGAAAA AAATCACTGG 480

ATATACCACC GTTGATATAT CCCAATGGCA TCGTAAAGAA CATTTTGAGG CATTTCAGTC 540

AGTTGCTCAA TGTACCTATA ACCAGACCGT TCAGCTGGAT ATTACGGCCT TTTTAAAGAC 600

CGTAAAGAAA AATAAGCACA AGTTTTATCC GGCCTTTATT CACATTCTTG CCCGCCTGAT 660

GAATGCTCAT CCGGAGTTCC GTATGGCAAT GAAAGACGGT GAGCTGGTGA TATGGGATAG 720

TGTTCACCCT TGTTACACCG TTTTCCATGA GCAAACTGAA ACGTTTTCAT CGCTCTGGAG 780

TGAATACCAC GACGATTTCC GGCAGTTTCT ACACATATAT TCGCAAGATG TGGCGTGTTA 840

CGGTGAAAAC CTGGCCTATT TCCCTAAAGG GTTTATTGAG AATATGTTTT TCGTCTCAGC 900

CAATCCCTGG GTGAGTTTCA CCAGTTTTGA TTTAAACGTG GCCAATATGG ACAACTTCTT 960

CGCCCCCGTT TTCACAATGG GCAAGTATTA TACGCAAGGC GACAAGGTGC TGATGCCGCT 1020

GGCGATTCAG GTTCATCATG CCGTTTGTGA TGGCTTCCAT GTCGGCAGAA TGCTTAATGA 1080

ATTACAACAG TACTGCGATG AGTGGCAGGG CGGGGCGTAA TTTTTTTAAG GCAGTTATTG 1140

GTGCCCTTAA ACGCCTGGTG CTACGCCTGA ATAAGTGATA ATAAGCGGAT GAATGGCAGA 1200

AATTCGTCGG ATCAAAAGGA TCTAGGTGAA GATCCTTTTT GATAATCTCA TGACCAAAAT 1260

CCCTTAACGT GAGTTTTCGT TCCACTGAGC GTCAGACCCC GTAGAAAAGA TCAAAGGATC 1320

TTCTTGAGAT CCTTTTTTTC TGCGCGTAAT CTGCTGCTTG CAAACAAAAA AACCACCGCT 1380

ACCAGCGGTG GTTTGTTTGC CGGATCAAGA GCTACCAACT CTTTTTCCGA AGGTAACTGG 1440

CTTCAGCAGA GCGCAGATAC CAAATACTGT TCTTCTAGTG TAGCCGTAGT TAGGCCACCA 1500

CTTCAAGAAC TCTGTAGCAC CGCCTACATA CCTCGCTCTG CTAATCCTGT TACCAGTGGC 1560

TGCTGCCAGT GGCGATAAGT CGTGTCTTAC CGGGTTGGAC TCAAGACGAT AGTTACCGGA 1620

TAAGGCGCAG CGGTCGGGCT GAACGGGGGG TTCGTGCACA CAGCCCAGCT TGGAGCGAAC 1680

GACCTACACC GAACTGAGAT ACCTACAGCG TGAGCATTGA GAAAGCGCCA CGCTTCCCGA 1740

AGGGAGAAAG GCGGACAGGT ATCCGGTAAG CGGCAGGGTC GGAACAGGAG AGCGCACGAG 1800

GGAGCTTCCA GGGGGAAACG CCTGGTATCT TTATAGTCCT GTCGGGTTTC GCCACCTCTG 1860

ACTTGAGCGT CGATTTTTGT GATGCTCGTC AGGGGGGCGG AGCCTATGGA AAAACGCCAG 1920

CAACGCGGCC TTTTTACGGT TCCTGGCCTT TTGCTGGCCT TTTGCTCACA TGTTCTTTCC 1980

TGCGTTATCC CCTGATTCTG TGGATAACCG TATTACCGCC TTTGAGTGAG CTGATACCGC 2040

TCGCCGCAGC CGAACGACCG AGCGCAGCGA GTCAGTGAGC GAGGAAGCGG AAGAGCGCTA 2100

GCAGCACGCC ATAGTGACTG GCGATGCTGT CGGAATGGAC GATACTTGTT ACCCATCATT 2160

GAATTTTGAA CATCCGAACC TGGGAGTTTT CCCTGAAACA GATAGTATAT TTGAACCTGT 2220

ATAATAATAT ATAGTCTAGC GCTTTACGGA AGACAATGTA TGTATTTCGG TTCCTGGAGA 2280

AACTATTGCA TCTATTGCAT AGGTAATCTT GCACGTCGCA TCCCCGGTTC ATTTTCTGCG 2340

TTTCCATCTT GCACTTCAAT AGCATATCTT TGTTAACGAA GCATCTGTGC TTCATTTTGT 2400

AGAACAAAAA TGCAACGCGA GAGCGCTAAT TTTTCAAACA AAGAATCTGA GCTGCATTTT 2460

TACAGAACAG AAATGCAACG CGAAAGCGCT ATTTTACCAA CGAAGAATCT GTGCTTCATT 2520

TTTGTAAAAC AAAAATGCAA CGCGAGAGCG CTAATTTTTC AAACAAAGAA TCTGAGCTGC 2580

ATTTTTACAG AACAGAAATG CAACGCGAGA GCGCTATTTT ACCAACAAAG AATCTATACT 2640

TCTTTTTTGT TCTACAAAAA TGCATCCCGA GAGCGCTATT TTTCTAACAA AGCATCTTAG 2700

ATTACTTTTT TTCTCCTTTG TGCGCTCTAT AATGCAGTCT CTTGATAACT TTTTGCACTG 2760

TAGGTCCGTT AAGGTTAGAA GAAGGCTACT TTGGTGTCTA TTTTCTCTTC CATAAAAAAA 2820

GCCTGACTTC ACTTCCCGCG TTTACTGATT ACTAGCGAAG CTGCGGGTGC ATTTTTTCAA 2880

GATAAAGGCA TCCCCGATTA TATTCTATAC CGATGTGGAT TGCGCATACT TTGTGAACAG 2940

AAAGTGATAG CGTTGATGAT TCTTCATTGG TCAGAAAATT ATGAACGGTT TCTTCTATTT 3000

TGTCTCTATA TACTACGTAT AGGAAATGTT TACATTTTCG TATTGTTTTC GATTCACTCT 3060

ATGAATAGTT CTTACTACAA TTTTTTTGTC TAAAGAGTAA TACTAGAGAT AAACATAAAA 3120

AATGTAGAGG TCGAGTTTAG ATGCAAGTTC AAGGAGCGAA AGGTGGATGG GTAGGTTATA 3180

TAGGGATATA GCACAGAGAT ATATAGCAAA GAGATACTTT TGAGCAATGT TTGTGGAAGC 3240

GGTATTCGC 3249

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1115 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Saccharomyces cerevisiae

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TCGACGGATC TGGCTTTTCA ATTCAATTCA TCATTTTTTT TTTATTCTTT TTTTTGATTT 60

CGGTTTCCTT GAAATTTTTT TGATTCGGTA ATCTCCGAAC AGAAGGAAGA ACGAAGGAAG 120

GAGCACGATT TTTGCATGGT ATATATACGG ATATGTAGTG TTGAAGAAAC ATGAAATTGC 180

CCAGTATTCT TAACCCAACT GCACAGAACA AAAACCGGAA ACGAAGATAA ATCATGTCGA 240

AAGCTACATA TAAGGAACGT GCTGCTACTC ATCCTAGTCC TGTTGCTGCC AAGCTATTTA 300

ATATCATGCA CGAAAAGCAA ACAAACTTGT GTGCTTCATT GGATGTTCGT ACCACCAAGG 360

AATTACTGGA GTTAGTTGAA GCATTAGGTC CCAAAATTTG TTTACTAAAA ACACATGTGG 420

ATATCTTGAC TGATTTTTCG ATGGAGGGCA CAGTTAAGCC GCTAAAGGCA TTATCCGCCA 480

AGTACAATTT TTTACTCTTC GAAGACAGAA AATTTGCTGA CATTGGTAAT ACAGTCAAAT 540

TGCAGTACTC TGCGGGTGTC TATAGAATAG CAGAATGGGC AGACATTACG AATGCACACG 600

GTGTGGTGGG CCCAGGTATT GTTAGCGGTT TGAAGCAGGC GGCAGAAGAA GTAACAAAGG 660

AACCTAGAGG ACTTTTGATG TTAGCAGAAT TGTCATGCAA GGGCTCCCTA TCTACTGGAG 720

AATATACTAA GGGTACTGTT GACATTGCGA AGAGCGACAA AGATTTTGTT ATCGGCTTTA 780

TTGCTCAAAG AGACATGGGT GGAAGAGATG AAGGTTACGA TTGGTTGATT ATGACACCCG 840

GTGTGGGTTT AGATGACAAG GGAGACGCAT TGGGTCAACA GTATAGAACC GTGGATGATG 900

TGGTCTCTAC AGGATCTGAC ATTATTATTG TTGGAAGAGG ACTATTTGCA AAGGGAAGGG 960

ATGCTAAGGT AGAGGGTGAA CGTTACAGAA AAGCAGGCTG GGAAGCATAT TTGAGAAGAT 1020

GCGGCCAGCA AAACTAAAAA ACTGTATTAT AAGTAAATGC ATCTATACTA AACTCACAAA 1080

TTAGAGCTTC AATTTAATTA TATCAGTTAT TACCC 1115

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1334 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Saccharomyces cerevisiae
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

AATTCCCATT ATTTAAGGAC CTATTGTTTT TTCCAATAGG TGGTTAGCAA TCGTCTTACT 60

TTCTAACTTT TCTTACCTTT TACATTTCAG CAATATATAT ATATATTTCA AGGATATACC 120

ATTCTAATGT CTGCCCCTAT GTCTGCCCCT AAGAAGATCG TCGTTTTGCC AGGTGACCAC 180

GTTGGTCAAG AAATCACAGC CGAAGCCATT AAGGTTCTTA AAGCTATTTC TGATGTTCGT 240

TCCAATGTCA AGTTCGATTT CGAAAATCAT TTAATTGGTG GTGCTGCTAT CGACGCTACA 300

GGTGTCCCAC TTCCAGATGA GGCGCTGGAA GCCTCCAAGA AGGTTGATGC CGTTTTGTTA 360

GGTGCTGTGG GTGGTCCTAA ATGGGGTACC GGTAGTGTTA GACCTGAACA AGGTTTACTA 420

AAAATCCGTA AAGAACTTCA ATTGTACGCC AACTTAAGAC CATGTAACTT TGCATCCGAC 480

TCTCTTTTAG ACTTATCTCC AATCAAGCCA CAATTTGCTA AAGGTACTGA CTTCGTTGTT 540

GTCAGAGAAT TAGTGGGAGG TATTTACTTT GGTAAGAGAA AGGAAGACGA TGGTGATGGT 600

GTCGCTTGGG ATAGTGAACA ATACACCGTT CCAGAAGTGC AAAGAATCAC AAGAATGGCC 660

GCTTTCATGG CCCTACAACA TGAGCCACCA TTGCCTATTT GGTCCTTGGA TAAAGCTAAT 720

GTTTTGGCCT CTTCAAGATT ATGGAGAAAA ACTGTGGAGG AAACCATCAA GAACGAATTT 780

CCTACATTGA AGGTTCAACA TCAATTGATT GATTCTGCCG CCATGATCCT AGTTAAGAAC 840

CCAACCCACC TAAATGGTAT TATAATCACC AGCAACATGT TTGGTGATAT CATCTCCGAT 900

GAAGCCTCCG TTATCCCAGG TTCCTTGGGT TTGTTGCCAT CTGCGTCCTT GGCCTCTTTG 960

CCAGACAAGA ACACCGCATT TGGTTTGTAC GAACCATGCC ACGGTTCTGC TCCAGATTTG 1020

CCAAAGAATA AGGTTGACCC TATCGCCACT ATCTTGTCTG CTGCAATGAT GTTGAAATTG 1080

TCATTGAACT TGCCTGAAGA AGGTAAGGCC ATTGAAGATG CAGTTAAAAA GGTTTTGGAT 1140

GCAGGTATCA GAACTGGTGA TTTAGGTGGT TCCAACAGTA CCACCGAAGT CGGTGATGCT 1200

GTCGCCGAAG AAGTTAAGAA AATCCTTGCT TAAAAAGATT CTCTTTTTTT ATGATATTTG 1260

TACATAAACT TTATAAATGA AATTCATAAT AGAAACGACA CGAAATTACA AAATGGAATA 1320

TGTTCATAGG GTAG 1334

CLAIMS.

1. Promoter DNA incorporating a structural gene starting position characterised in that the DNA has a unique SspI restriction site at the structural gene start position.

2. A method for producing a DNA as claimed in claim 1 comprising subjecting promoter DNA to Site-Directed Mutagenisis to create a unique SspI restriction site at the structural gene start position.

3. A method as claimed in claim 2 wherein the position of the created site is such that the triplet sequence, ATG, corresponding to the translational start codon of the structural gene becomes ATA within the SspI recognition site AATATT.

4. A method as claimed in claim 2 or 3 wherein the heterologous gene to be inserted is similarly modified wherein the nucleotide triplet corresponding to the translational start codon is changed to CAG, while the triplet immediately 5' is changed to CTG in order to create a PstI restriction site, CTGCAG.

5. A method as claimed in claim 4 wherein the creation of the PstI, or equivalent site, is performed simultaneously to isolation of the gene by utilising a mutagenic primer in a polymerase chain reaction (PCR) catalysed gene amplification procedure.

6. A method as claimed in claim 4 or 5 wherein the heterologous gene is digested with PstI restriction endonuclease and the 3' overhanging ends removed by the 3' to 5' exo-nucleolytic activity of T4 DNA polymerase, the gene then excised using one or more of the restriction enzymes whose sites are present within the polylinker of the vector whereby the first base of the blunt-ended DNA fragment is the third nucleotide, "G", of its first codon and the gene DNA is then ligated into the vector which has been digested previously with SspI and a restriction enzyme compatible with that used to excise the heterologous gene, whereby fusion of the vector promoter region (which ends in "AT") and heterologous gene (which begins in a "G") results in the recreation of the translational start, ATG.

7. Recombinant DNA comprising a yeast promoter sequence characterized in that the leader region of the promoter sequence is replaced with leader sequence of the replication protein 2 (REP2) gene (ORF C) of the yeast 2 μm plasmid.

8. Recombinant DNA as claimed in claim 7 wherein the yeast promoter derived portion is that of the phosphoglycerate kinase (PGK) promoter.

9. Recombinant DNA as claimed in claim 7 or 8 wherein the upstream activating sequence element and TATA-box are those as found in the PGK promoter.

10. Recombinant DNA as claimed in any one of claims 7 to 9 wherein the UAS element and TATA-box are fused to the 86 nucleotides residing immediately 5' to the 2μm plasmid REP2 gene.

11. Recombinant DNA comprising a sequence of bases 1 to 635 of SEQ ID 1.

12. An expression cassette comprising recombinant DNA as claimed in any one of claims 1 and 7 to 11 characterized in that it further includes a copy of the lacZ' gene, containing the multiple cloning sites of pMTL23, preceded by the promoter DNA of any one of claims 7 to 11, and followed by tandemly arranged, yeast gene-derived, transcriptional terminators.

13. An expression cassette comprising a DNA sequence SEQ ID 1.

14. A method for cloning a heterologous gene into an expression cassette as claimed in claim 12 or 13 wherein a primer oligonucleotide for the heterologous gene is designed having its 5' end corresponding to the G residue of the ATG translational start point, and a specific sequence amplification is carried out using the primer oligonucleotide to isolate the heterologous gene ready for insertion into the cassette.

15. An *E. coli* or *S. cerevisiae* shuttle plasmid comprising an expression cassette as claimed in claim 12, or 13 or as provided by a method as described in any one of claims 2 to 6 or 14.

Fig. 1.

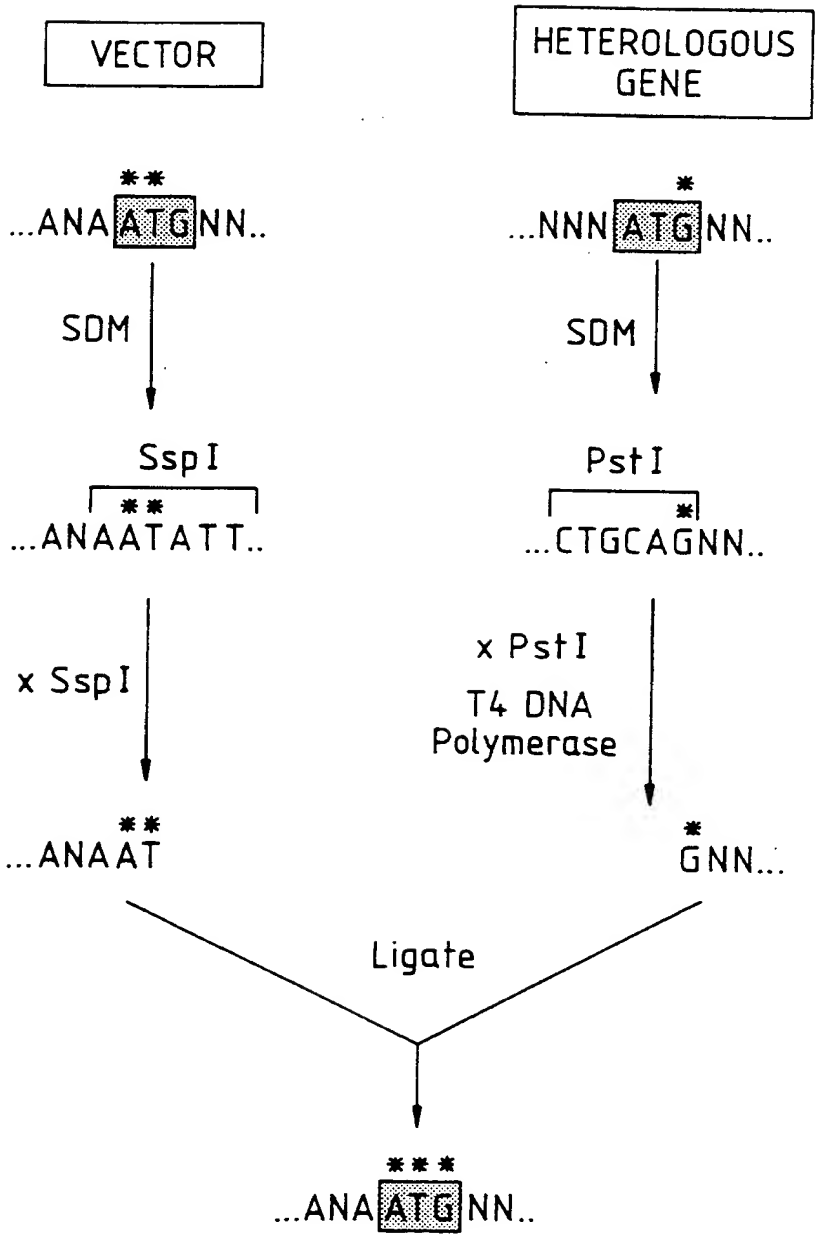

VECTOR
HETEROLOGOUS GENE
...ANAATGNN..
...NNNATGNN..
SDM
SDM
SspI
PstI
...ANAATATT..
...CTGCAGNN..
x SspI
x PstI
T4 DNA Polymerase
...ANAAT
GNN...
Ligate
...ANAATGNN..

Fig.2.

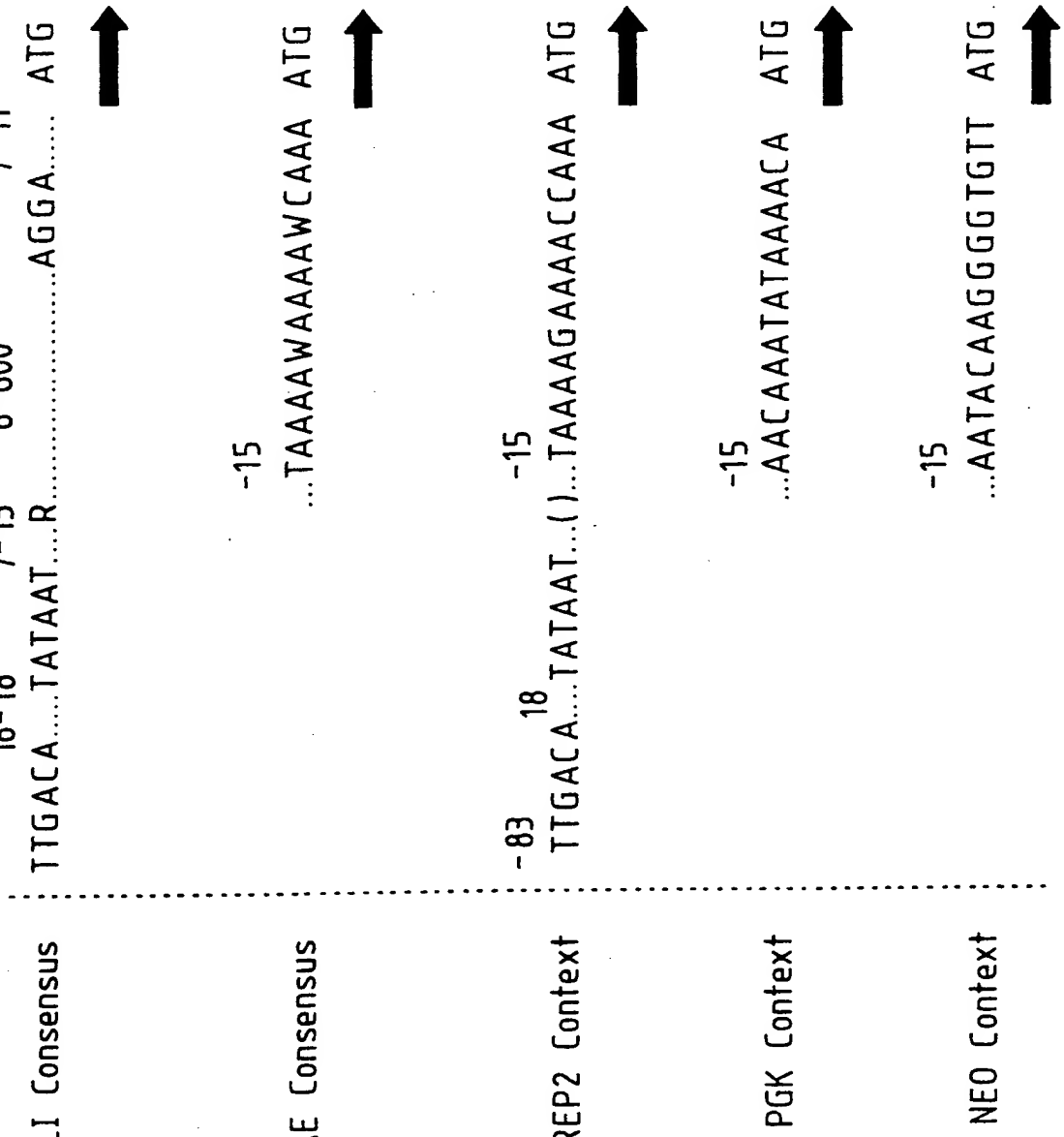

E.COLI Consensus
-35 -10
16-18 7-15 6-600 7-11
TTGACA.....TATAAT....R.....AGGA..... ATG
S. CEREVISIAE Consensus
-15
...TAAAAWAAAAWCAAA ATG
REP2 Context
-83 18 -15
TTGACA....TATAAT...( )...TAAAAGAAAACCAAA ATG
PGK Context
-15
...AACAAATATAAAACA ATG
NEO Context
-15
...AATACAAGGGGTGTT ATG

Fig. 3.

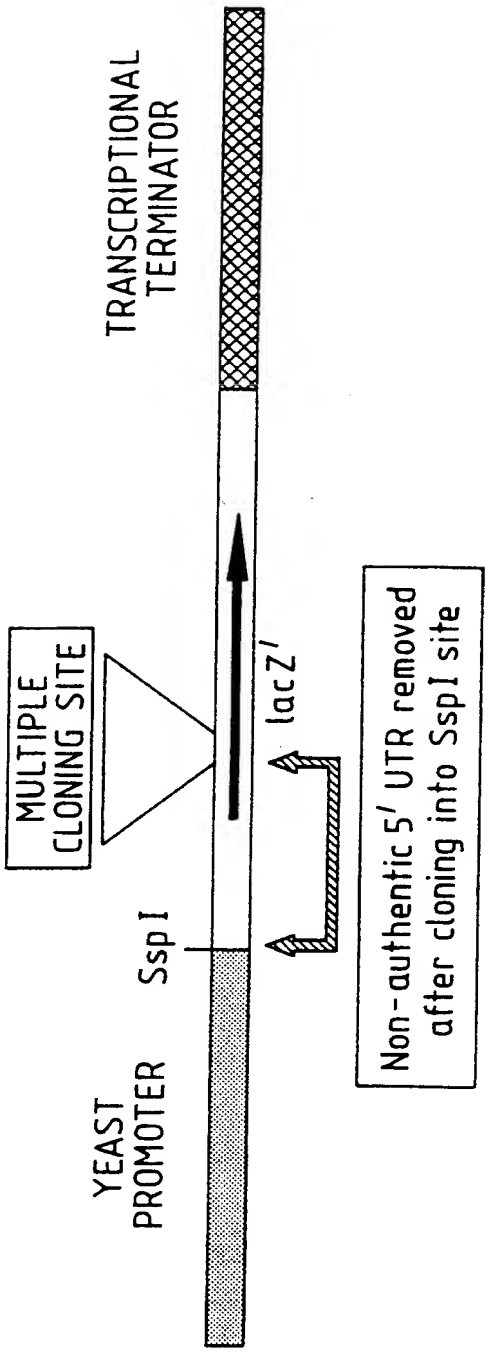

TRANSCRIPTIONAL TERMINATOR
MULTIPLE CLONING SITE
lacZ'
Non-authentic 5' UTR removed after cloning into SspI site
YEAST PROMOTER
SspI

Fig. 4.

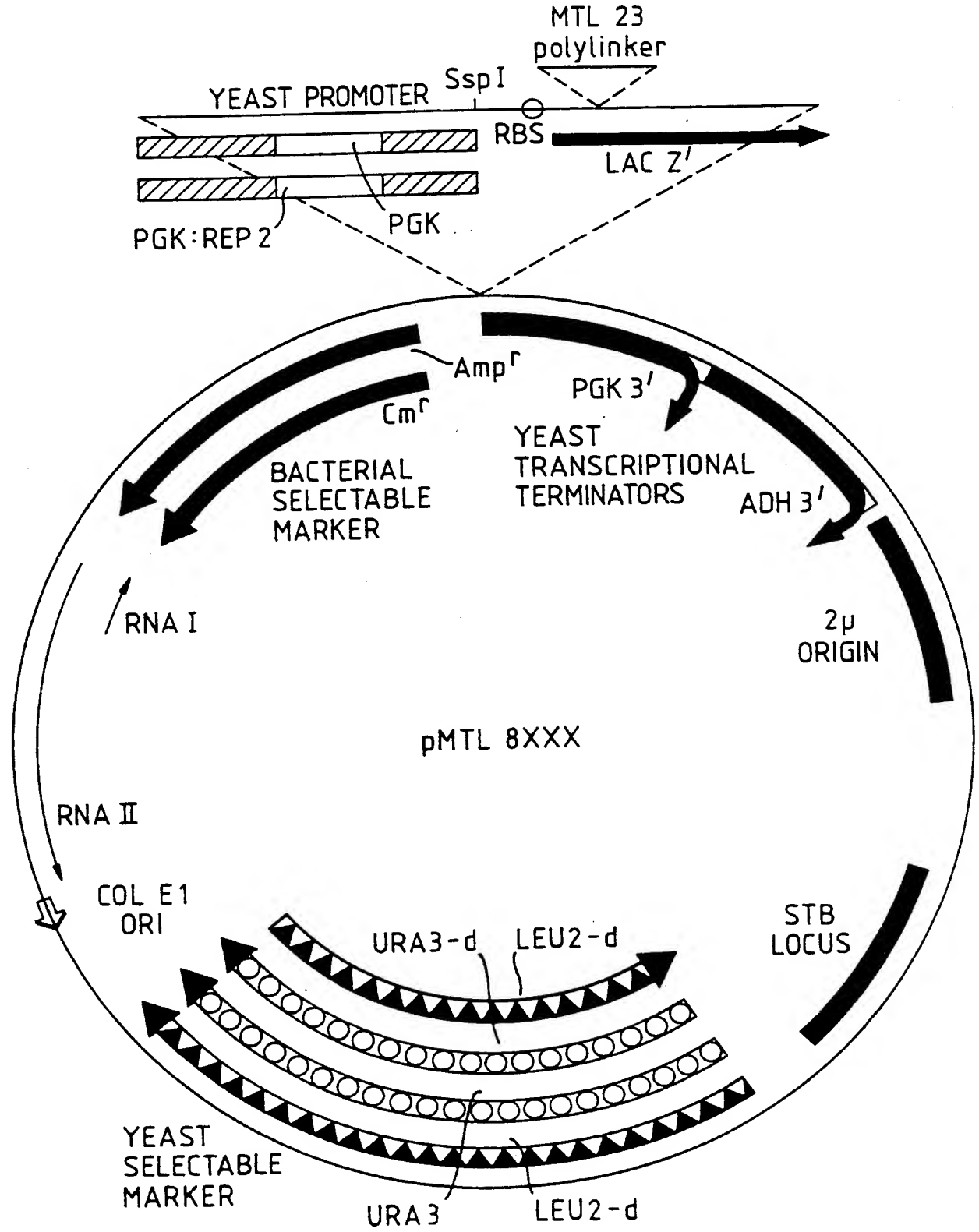

MTL 23
polylinker
YEAST PROMOTER
SspI
RBS
LAC Z'
PGK
PGK:REP 2
Amp^r
PGK 3'
Cm^r
YEAST
TRANSCRIPTIONAL
TERMINATORS
BACTERIAL
SELECTABLE
MARKER
ADH 3'
RNA I
2μ
ORIGIN
pMTL 8XXX
RNA II
COL E1
ORI
STB
LOCUS
URA3-d
LEU2-d
YEAST
SELECTABLE
MARKER
URA 3
LEU2-d

Fig. 5.

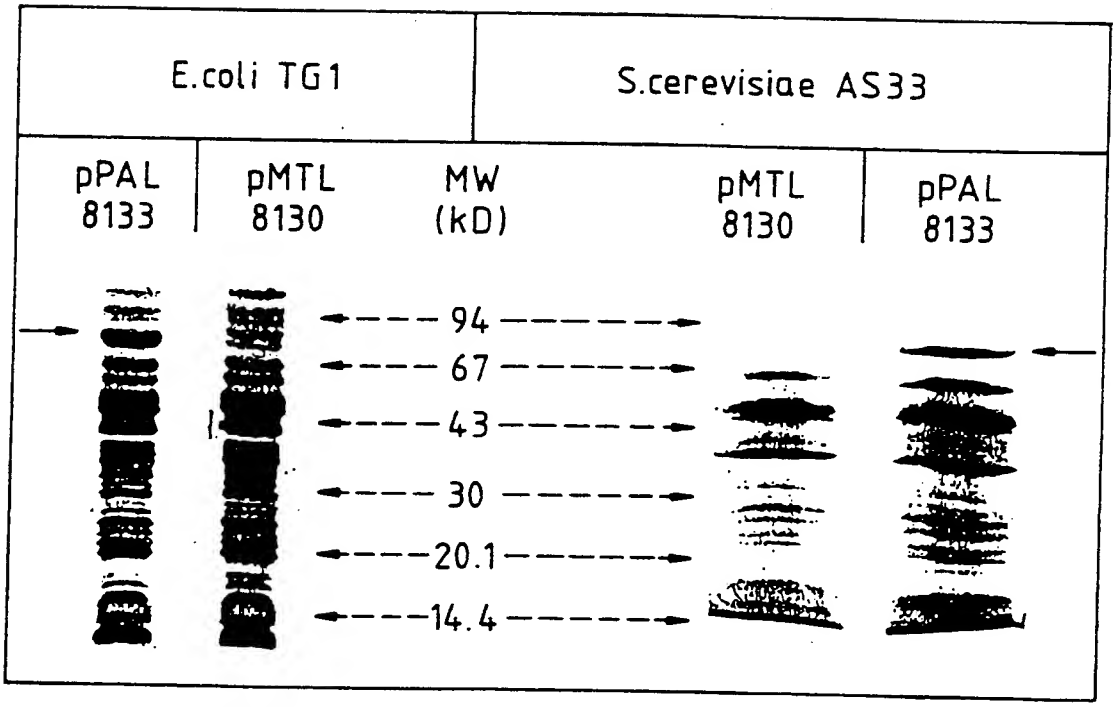

E.coli TG1
S.cerevisiae AS33
pPAL 8133
pMTL 8130
MW (kD)
pMTL 8130
pPAL 8133
94
67
43
30
20.1
14.4

Fig.6.

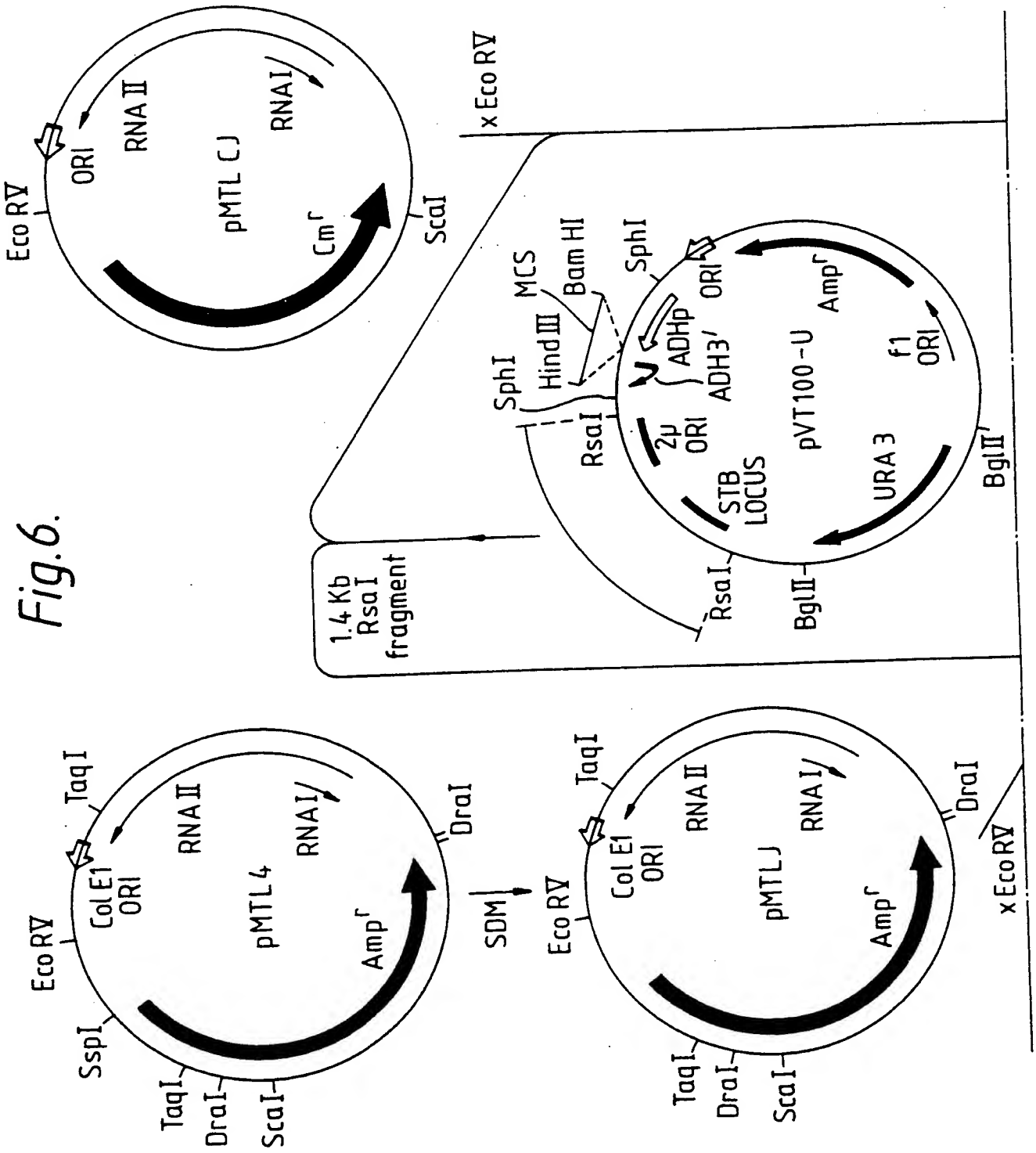
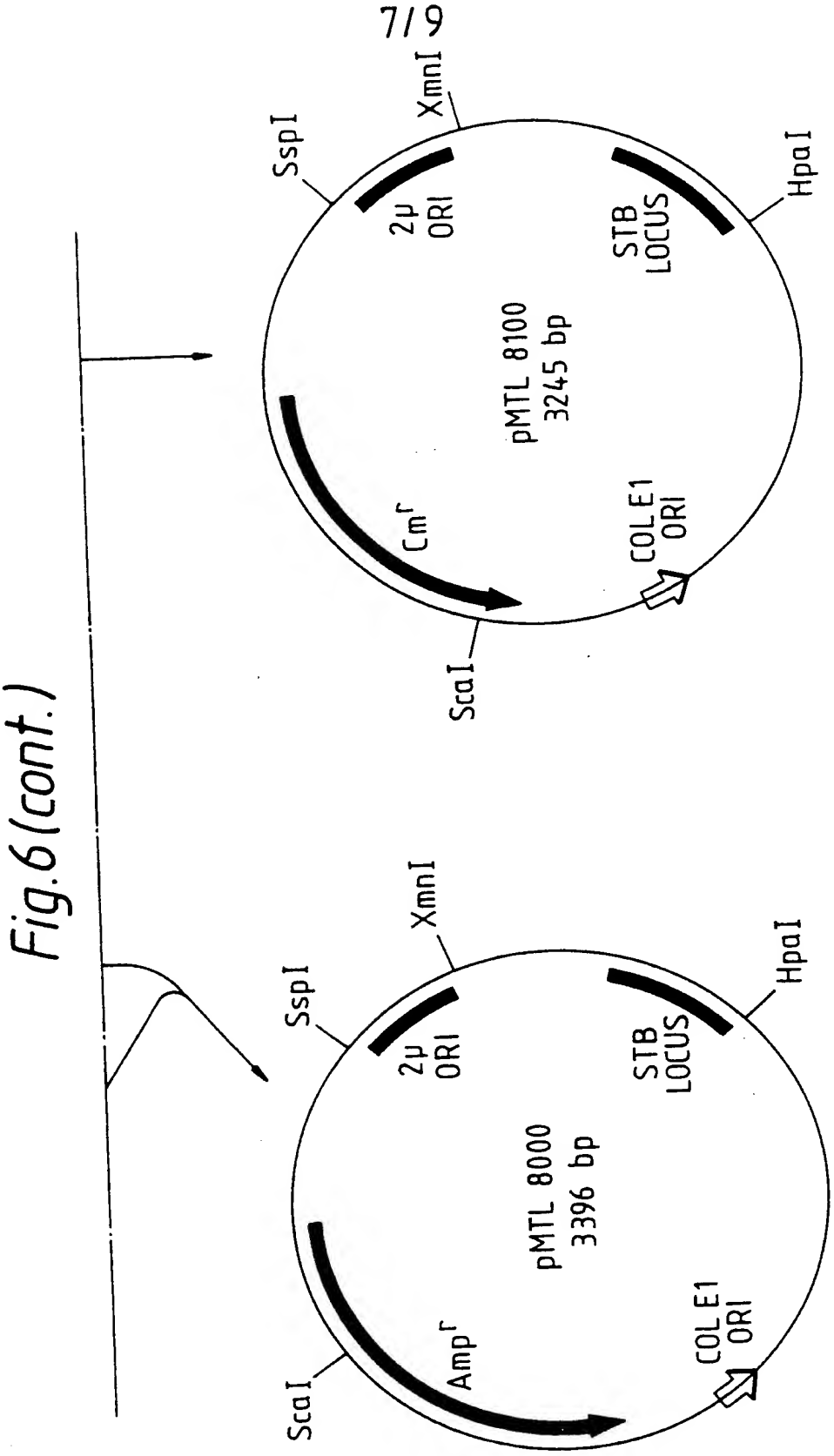

Eco RV
ORI
RNA II
pMTL CJ
RNA I
Cm^r
Sca I
x Eco RV
Sph I
Hind III
MCS
Bam HI
Sph I
ADHp
ADH3'
ORI
Amp^r
pVT100-U
f1 ORI
2μ ORI
STB LOCUS
URA 3
Rsa I
Bgl II
Bgl II'
1.4 Kb Rsa I fragment
Taq I
Col E1 ORI
RNA II
RNA I
Dra I
Eco RV
Ssp I
Taq I
Dra I
Sca I
pMTL 4
Amp^r
SDM
Eco RV
Taq I
Col E1 ORI
RNA II
RNA I
Dra I
pMTL J
Amp^r
x Eco RV
Taq I
Dra I
Sca I

Fig.6(cont.)


SspI
XmnI
HpaI
2μ ORI
STB LOCUS
pMTL 8100
3245 bp
Cmr
COLE1 ORI
ScaI
SspI
XmnI
HpaI
2μ ORI
STB LOCUS
pMTL 8000
3396 bp
Ampr
COLE1 ORI
ScaI

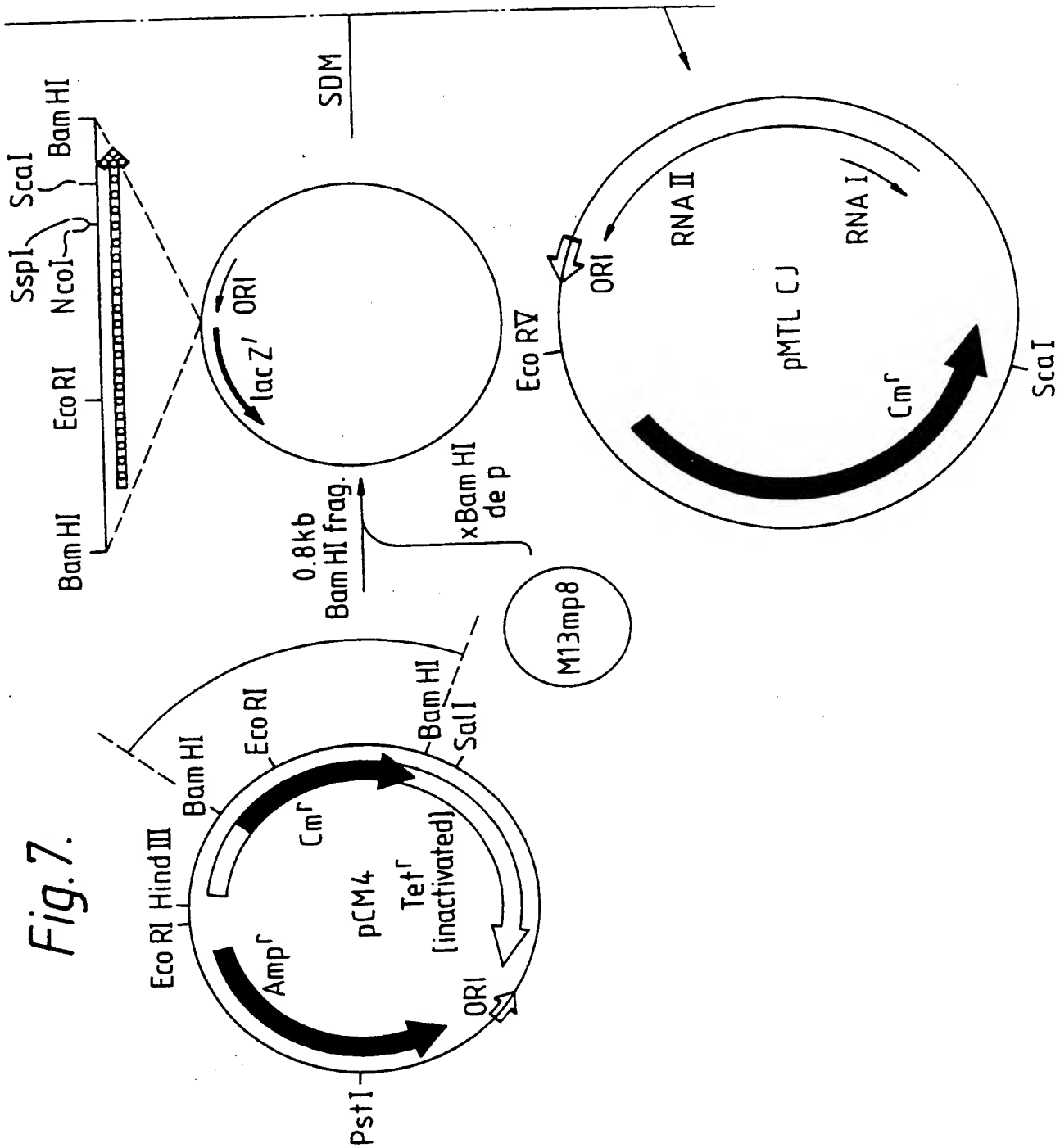
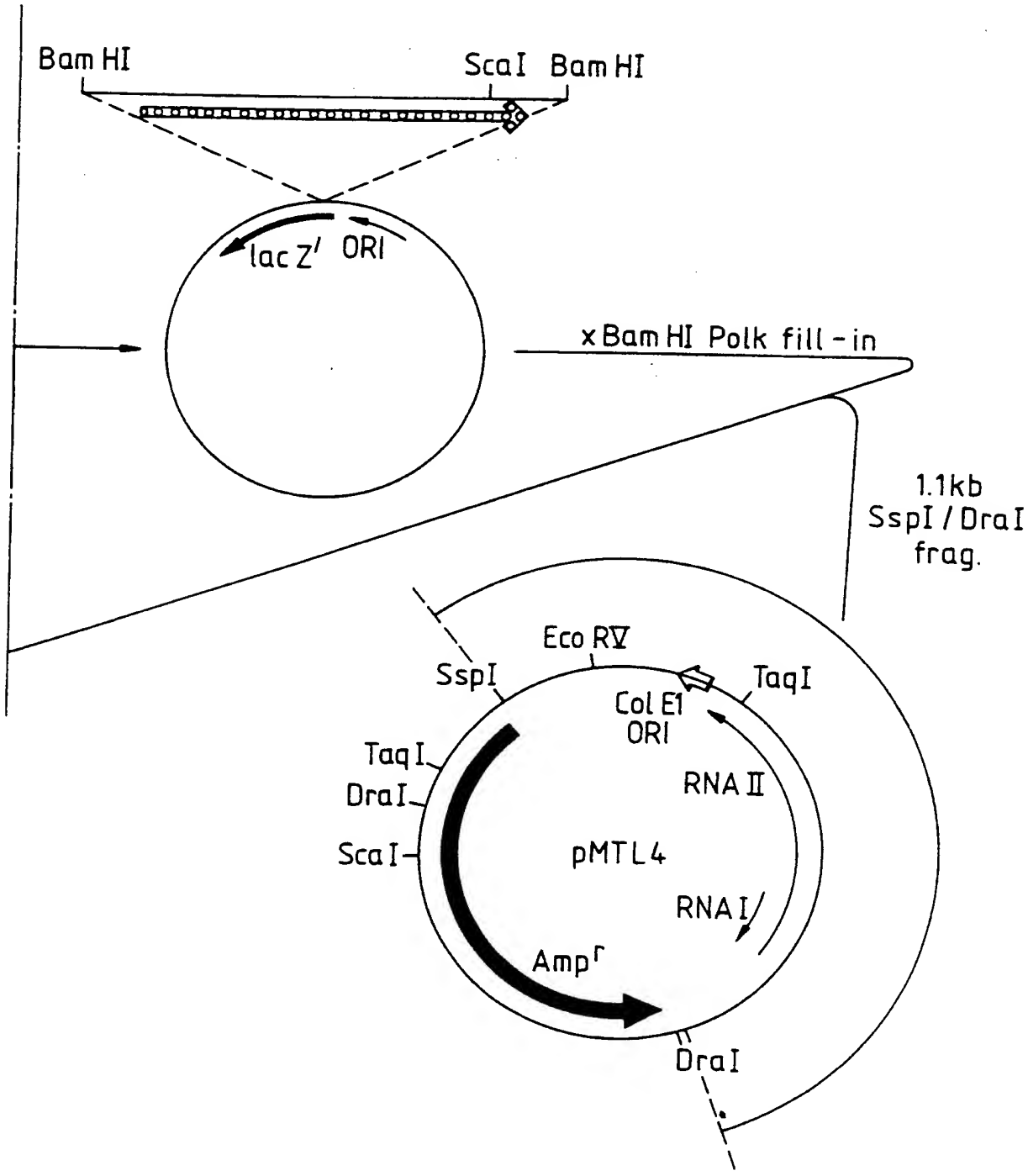

SDM
Bam HI
Sca I
Ssp I
Nco I
Eco RI
Bam HI
lacZ'
ORI
0.8kb
Bam HI frag.
x Bam HI
de p
M13mp8
Eco RV
ORI
RNA II
RNA I
pMTL CJ
Cm^r
Sca I
Eco RI Hind III
Bam HI
Eco RI
Cm^r
Bam HI
Sal I
pCM4
Amp^r
Tet^r
(inactivated)
ORI
Pst I

Fig. 7.

Fig.7(cont.)


Bam HI
Sca I Bam HI
lac Z' ORI
x Bam HI Polk fill-in
1.1kb
Sspl / Dra I
frag.
Eco RV
Sspl
Taq I
Col E1
ORI
Taq I
Dra I
RNA II
Sca I
pMTL4
RNA I
Amp^r
Dra I